SECTION MEETINGS

BALTIMORE
    Army Chemical Center   October 31, 1952
CLEVELAND
    University Hospitals   November 13, 1952
DISTRICT OF COLUMBIA
    Walter Reed Army Medical Center   December 4, 1952
IOWA
    State University of Iowa   November 18, 1952
MINNESOTA
    Mayo Foundation   October 24, 1952
NEW YORK
    New York Academy of Medicine   December 15, 1952
OHIO VALLEY
    Purdue University   October 25, 1952
ROCKY MOUNTAIN
    University of Colorado Medical School   November 8, 1952
SOUTHEASTERN
    University of Virginia   November 29, 1952
SOUTHWESTERN
    School of Aviation Medicine   November 7-8, 1952
WESTERN NEW YORK
    University of Rochester   November 15, 1952

Dehydroepiandrosterone* and Similar Substances in Urine of Premature Infants.† (19943)

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Dirscherl and Zilliken(2), using a modification of the Kober test for estrogens(3), described a simple color test (the "blue test") for dehydroepiandrosterone (DHA) and other closely related compounds. Patterson(4), Allen, Hayward, and Pinto(5), and Gardner and Migeon(6) have utilized this colorimetric procedure on urinary steroids to distinguish between patients with adrenal tumors and

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those with congenital adrenal hyperplasia. Among the 11 patients with adrenal tumor reported by the above authors, the test was positive 10 times. In the instance in which it was negative an abnormally large amount of DHA was found and it was stated that the typical blue color was obscured by contaminating yellow colors. Among the 15 patients with congenital adrenal hyperplasia, the test was always negative. The authors concluded that this is a valuable test to aid in differentiating between these conditions, particularly when it is positive.

It is the purpose of this communication to point out that the test is frequently positive in premature infants and in children under one year of age who have no evidence of an adrenal tumor.

Methods and material. 17-ketosteroids were determined by the method of Bongiovanni (7). For the Zimmerman reaction, DHA was used for the standard.‡ The "blue test" for DHA was performed according to the method of Gardner and Migeon(6) on part of the urinary extract obtained for the Zimmerman reaction. Absorption curves were done on the Beckman spectrophotometer using a 1 cm light path. Allen(8) states that this color reaction can be used quantitatively, but for the purposes of this communication a qualitative "yes" or "no" answer was all that was necessary. Urine was collected from premature infants of various ages and weights, and receiving various diets, who were hospitalized because of prematurity. Male infants had a rubber tube taped to the penis. Females were catheterized. Urine was collected in bottles containing 5 ml of concentrated hydrochloric acid. Occasionally 24-hour collections were made, but usually random samples were collected. The urine of the infants under one year of age who were not premature was collected without preservative. These patients were hospitalized for a variety of conditions.

Results. The urine of 9 out of 18 premature infants yielded a positive "blue test" at least once. Of the urines of those infants with negative tests, 5 were tested only once; the

‡ Kindly supplied by the Schering Corp., Bloomfield, N. J.

remaining 4 were tested at least 3 times. Except for 2 infants who had positive tests on their only examination, the remaining infants had both positive and negative tests. Out of 66 examinations of the urine of these 18 infants, the "blue test" was positive 24 times. The test, when positive, seemed to be so in a random manner. Specimens of urine from the same child on different days, and at different times during the same day, would be either positive or negative. We could note no correlation between age, weight, diet, medication, time of day, or any stress factors, and the presence of a positive test. Nor was there any correlation between a positive "blue test" and the total amount of 17-ketosteroids. A 20 ml aliquot of urine containing as little as 0.05 mg of 17-ketosteroid was positive, and a sample containing 0.76 mg per 20 ml urine was negative. Conversely, a sample containing 0.58 mg was positive, and one containing 0.02 mg was negative. It has not yet been determined whether a negative test in the presence of relatively large amounts of 17-ketosteroids is due to interference with the production of the typical color by these steroids or contaminants, or whether there is an absolute decrease in the amount of DHA or DHA-like substances.

In 18 examinations of the urine of 15 patients under one year of age who were not prematures, but who were in the hospital for a variety of conditions, the test was positive once in 2 patients. One of these patients was recuperating from meningococcus meningitis, and the other had idiopathic hypoproteinemia.

The spectral transmission curves of many of these positive tests were studied. Fig. 1 shows a representative curve of a positive test with peaks at 375 mμ, 475 mμ, and a maximum at 605 mμ. On the same graph is a representative curve of a negative test, with peaks at 375 mμ and 475 mμ followed by a gentle slope without any peak at 605 mμ. The transmission curve of pure DHA has a peak at about 385, and a maximum peak at 605.

When urine giving a negative test was mixed with urine giving a positive test, the blue color appeared. When pure DHA was added to urine giving a negative test, an

FIG. 1.

hermaphrodite who was less than two months old.

The obvious morphological differences between the adrenal gland of the newborn and the older child or adult suggest that functional differences also may be present. This has been demonstrated by Klein(9) who showed that when ACTH is given to a premature infant, a sodium diuresis and potassium retention occurs. The opposite effect of ACTH administration occurs in older children or adults(9).

To our knowledge, our observation is the first evidence that steroid excretion in the premature infant may be qualitatively different from that of more mature individuals.

Summary. Examination of the urine of premature infants and children less than a year of age for DHA or DHA-like substances by means of a colorimetric test disclosed that in the absence of an adrenal tumor the test was positive at least once in half of the patients. Of all the urine samples collected from patients in this age group, the test was positive 30% of the time.

absorption curve was obtained which appeared to be virtually identical with that of a positive test. Therefore, it seems probable that the main compound giving the blue color is DHA.

Discussion. These observations oblige us to conclude that the test cannot be used to differentiate between an adrenal tumor and benign hyperplasia in premature infants; furthermore, it must be interpreted with caution in children under a year of age. The observations do not, of course, invalidate the conclusions of other authors concerning patients in the older age groups, and it may also be true, that the test will never be positive in children with the adrenogenital syndrome, even when they are very young. On two occasions the test was negative in a pseudo-

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Inability of Growth Hormone to Prevent the Anemia which Follows Hypophysectomy.* (19944)

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As part of a general reinvestigation of the entire problem of the endocrine control of red blood cell production, the effect of growth hormone on erythropoiesis has been investigated. When it was shown that after hypophysectomy there was a decrease in the concentration of red cells in the blood(1), hypoplasia of the erythrogenic elements of the bone marrow(2) and a decrease in the circulating red cell volume(3), it was necessary to determine which of the various hormones of the pituitary was responsible for maintaining red cell production at a normal level. To determine the importance of pituitary hormones in erythropoiesis one either removes a target organ, administers the target organ hormone, or administers the pituitary hormone. However, as growth hormone has no known target organ through which it acts, one can evaluate the importance of this hormone in maintaining the red cell volume only by the administration of growth hormone. Meyer, Stewart, Thewlis, and Rusch(4) reported that the administration of growth hormone to hypophysectomized rats for periods up to 9 weeks was invariably attended by reticulocytosis and bone marrow hyperplasia, but no increase in concentration of hemoglobin or erythrocytes. Boiling destroyed both the growth activity and the reticulocyte stimulating activity of the extract. Injection of growth hormone into normal rats produced no changes in the levels of reticulocytes, hemoglobin or erythrocytes. Overbeek(5,6) found no influence on the reticulocyte count of hypophysectomized rats after administration of growth hormone. Gaebler and Mathies(7) reported that the hemoglobin values of hypophysectomized rats injected with 1 mg growth hormone daily actually decreased during the treatment.

Because of this lack of agreement regarding the effect of growth hormone on erythropoiesis, an experiment was done to determine whether administration of growth hormone would prevent the decrease in circulating red cell volume which follows hypophysectomy.

Material and methods. Female rats of the Long-Evans strain (21 days of age) were placed in 3 groups of 17 animals each. The rats in one group were hypophysectomized, those in another were hypophysectomized and injected intraperitoneally with 1.25 mg of growth hormone daily for 30 days, and those in a third group were kept as normal controls. The growth hormone preparation used was potent in the standard 4-day tibia test when given at a daily dose of 0.025 mg. It was free from adrenocorticotropic hormone and follicle stimulating hormone, but contained some interstitial cell stimulating hormone and barely detectable traces of thyrotropic hormone at the 1.25 mg daily dose given for 30 days. Twenty-four hours after the last injection, the circulating red cell volumes were determined by the Fe^{59} labeled red cell dilution method (8).
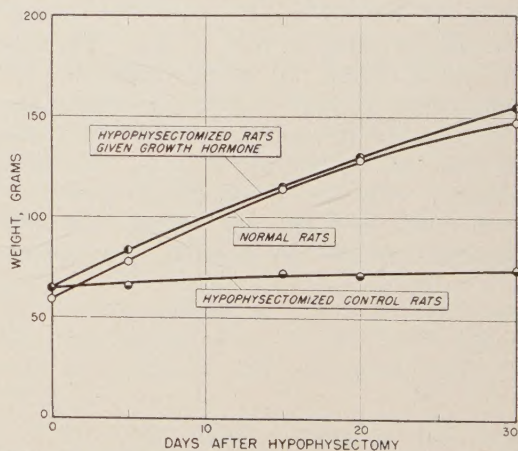

FIG. 1. Comparison of the average increase in body wt of normal rats, hypophysectomized rats, and hypophysectomized rats given growth hormone from the time of hypophysectomy.

TABLE I. Hematological Data. 17 animals in each group.

Group	Body wt, g	Hematocrit, %	Hemoglobin, g/100 ml	Red blood cell vol/100 g body wt, ml	Red blood cells $\times 10^6$, mm³
Hypophysectomized given 1.25 mg growth hormone daily for 30 days	156 ± 16*	37.7 ± 1.7	11 ± .8	1.78 ± .15	5.11 ± 1.17
Hypophysectomized controls	70 ± 7	39.5 ± 2.7	11.2 ± .7	1.87 ± .14	5.66 ± .61
Normal controls	149 ± 16	44.6 ± 2.1	13.7 ± 1.9	2.47 ± .16	6.02 ± .72

$$* \text{ Stand. dev.} = \sqrt{\frac{\Sigma (x)^2}{(N-1)}}.$$

The labeled cells were obtained from a Long-Evans donor previously injected with Fe^{59}. The experimental animals were injected through the saphenous vein with 0.1 ml of donor blood containing 0.03 μc Fe^{59} in the erythrocytes. After allowing 6 minutes for the blood to mix, the abdomen was opened and a sample of blood was drawn from the vena cava into a heparinized syringe. A known volume of this blood was pipetted into a vial and its activity counted directly in a scintillation counter(9). The total blood volume was calculated from the fraction of the injected activity recovered in this sample. The hematocrits were determined in Wintrobe tubes. The total blood volume thus calculated multiplied by the hematocrit gave the total circulating red cell volume. The values for the total circulating red cell volumes were then divided by the values for the body weights of the animals and the results are presented in terms of ml of red blood cells per 100 g body weight. The hemoglobin concentration was determined by the method of Turner(10). The adrenals, thyroids and testes were weighed and sectioned in order to show the completeness of hypophysectomy and to serve as a biological assay of the injected growth hormone.

Results. The weight gain of the 3 experimental groups is plotted in Fig. 1. It will be seen that the dose of growth hormone given to the hypophysectomized animals was sufficient to maintain a normal rate of growth. The hematological data are presented in Table I. The hypophysectomized controls (uninjected) developed the typical post-hypophysectomy anemia during the course of the experiment. The circulating red cell volume was 1.86 cc per 100 g body weight as compared to that of normal controls where there were 2.48 cc per 100 g. The hypophysectomized rats injected with growth hormone sufficient to maintain a normal rate of growth throughout the experiment (30 days) developed the same degree of anemia as the uninjected hypophysectomized controls.

Summary. In the search for the factor in the anterior pituitary responsible for maintenance of the circulating red cell volume, the effect of growth hormone in maintaining the red cell volume of hypophysectomized rats was investigated. Administration of doses of growth hormone sufficient to maintain a normal rate of growth for 30 days failed to prevent the development of the anemia which characteristically follows hypophysectomy.

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Influence of Tetraethylthiuram Disulfide (Antabuse)* on Duration of Action of Thiopental.   (19945)

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It has been established that severe liver dysfunction in the mouse, rat, and man(1,2) results in a significant prolongation of action of thiopental.   Giarman *et al.*(3) have reported a 60-fold increase in the duration of action of this drug in mice as a result of pretreatment with tetraethylthiuram disulfide (Antabuse).   In view of the known inhibitory effects of this latter compound on xanthine oxidase(4), Giarman *et al.* have suggested the possibility that this enzyme may function in the metabolism of thiopental to its carboxylic acid derivative(5).   If such is the case, it should be possible to demonstrate such an action of xanthine oxidase *in vitro* and an important step in the metabolism of thiopental would have been established.   Prior to the institution of such *in vitro* studies it was felt that the experiments of Giarman *et al.*(3) should be repeated and extended to animal species other than the mouse.   The following report is the result of such studies in the mouse, rat, and dog.

Methods and results.   In Table I are summarized the data obtained from experiments on 5 groups of mice.   Animals in group A received 1 g/kg/day of Antabuse orally as a 10% suspension in peanut oil.   On the day following the last of 3 such administrations, these mice and 11 controls received 30 mg/kg of sodium thiopental intravenously.   Duration of action was determined as the time elapsing between loss and return of the righting reflex.

In the remaining 4 groups each animal served as his own control and received the same dose of thiopental as did those animals in group A.   Thiopental was first administered one to 6 days before Antabuse and again 24 hours after the last dose of Antabuse.   Groups B and C received Antabuse (1 g/kg) orally as a 10% suspension in peanut oil on 3 successive days.   Group D received 25 mg of Antabuse (aqueous suspension) per day for 3 days.   Animals in Group E received the same dose of Antabuse as animals in groups A, B, and C except that the drug was suspended in a solution of tragacanth and administered for 5 days.   In none of these groups was there a significant prolongation of the action of thiopental (Table I).

Five mongrel dogs received 100 mg/kg of Antabuse (orally in capsules) daily for 3 successive days.   Six days prior to the administration of Antabuse and 24 hours after the last dose of this drug each animal received 20 mg/kg of sodium thiopental intravenously and its duration of action determined as the time elapsing between the loss and return of the righting reflex.   It is apparent from the data in Table II that there is no statistically significant difference between the values before and after Antabuse treatment ($p > 0.4$).

Three groups of rats (Table III) received a single dose of 1 g/kg of Antabuse‡ (10% suspension in peanut oil) by the oral route.   Three to 5 days prior to the administration

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* Antabuse, tetraethylthiuram disulfide, was supplied by Ayerst, McKenna and Harrison, Ltd.

† Present address: University of Wisconsin, Madison, Wisc.

‡ Daily administrations of Antabuse at a dose level of 1 g/kg for 3 days were lethal to all 10 rats in one group, therefore, a single dose of 1 g/kg was employed.

TABLE I. Effect of Antabuse on Duration of Action of Thiopental in Mice.

Group	Strain	No. animals	Control	After Antabuse	p
A	Rockefeller N.I.H.	11	6	—	>.1
		10	—	22	
B	Swiss albino	10	4.3	7.4	>.2
C	Rockland "All purpose"	7	4	5.5	>.05
D		7	5.1	3.5	>.02
E		9	9.3	7.3	>.05

Mean duration of action (min.) spans the Control / After Antabuse columns.

TABLE II. Effect of Antabuse on Duration of Action of Thiopental in Dogs.

	Duration of action (min.)	
Wt, kg	Control	After Antabuse
14	42	37
10.8	57	61
8.7	50	50
9.5	44	43
8.8	77	104
Avg	54	59

TABLE III. Effect of Antabuse on Duration of Action of Thiopental in Rats.

Group	No. animals	Control	After Antabuse	p
A	8	27.1	171.4	<.001
B	6	18.3	34.7	<.001
C	4	47.5	90	<.02

Mean duration of action (min.) spans the Control / After Antabuse columns.

of Antabuse and again 24 hours after the drug, each animal received 25 mg/kg of sodium thiopental, either intravenously (groups A and B) or intraperitoneally (group C). The duration of action of thiopental was significantly increased in each of the 3 groups of rats by the administration of Antabuse (Table III).

Blood and brain levels of thiopental at the time of return of the righting reflex were determined in 2 groups of rats after the intravenous administration of 25 mg/kg of sodium thiopental. One group of animals received no Antabuse and the second group (animals from group B above) received Antabuse 24 hours previously. Thiopental was determined in aliquots of brain and blood by the method of Brodie et al.(5). The results revealed a suggestive but not significant difference between the average blood levels of the 2 groups, the blood levels of thiopental in the Antabuse treated animals at the return of the righting reflex being slightly lower than in the control animals. There was no significant difference in the brain levels between the 2 groups.

Discussion. At the present time we are unable to offer any explanation for our inability to substantiate the results of Giarman et al.(3) in the mouse since, as far as we can determine, the experiments in our laboratory were performed in exactly the same manner as those reported by Giarman. Although it has been suggested(3) that Antabuse may interfere with the metabolism of thiopental in the mouse by virtue of an inhibitory action on xanthine oxidase, such an explanation does not seem likely in the case of the rat. A more logical explanation is suggested by the experiments of Fitzhugh et al.(6,7). In chronic experiments with mice, rats, and dogs, these workers observed no signs of central nervous system pathology in the mouse or dog, whereas such signs were rather severe in the rat. No histopathology was observed in the brains of dogs treated with Antabuse. However, rats, placed on a diet containing 2500 ppm of Antabuse, evidenced areas of calcification of the brain in 2 to 4 months. Such results suggest that the central nervous system of the rat is more susceptible to the effects of Antabuse than that of either the mouse or the dog. If it is assumed that this drug prolongs the action of thiopental by virtue of some alteration in the sensitivity of the central nervous system to the thiobarbiturates, then Fitzhugh's observations may offer an explanation for the species difference we have observed.

Such a central action of Antabuse also has been suggested by Graham et al.(8) as an explanation for the prolongation of the action of phenobarbital and hexobarbital in the rat and guinea pig by this drug.

Summary. The influence of Antabuse on the duration of action of thiopental has been determined in 3 animal species: mice, rats, and dogs. Oral administration of Antabuse at a dose level of 1 g/kg/day for as long as 5 days in the mouse or 100 mg/kg/day for 3 days in the dog did not affect the duration of action of anesthetic doses of thiopental. However, single oral doses of 1 g/kg of Antabuse in the rat resulted in a significant prolongation of action. These results are discussed in relation to the possible site of action of Antabuse and it is suggested that the effect in the rat may be due to a central nervous system action of the drug.

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A Simple Procedure for Preparing Anaerobic Plasma.   (19946)

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In the determination of blood gases it is frequently desired to obtain samples of plasma which have not been exposed to air. The following method has proven very satisfactory in our laboratory. It has the particular advantages that: (a) small samples can be handled in this way, and (b) the plasma samples can be readily freed from any trace of mineral oil which may be present.

Procedure. The apparatus consists of the following special pieces: (a) a tuberculin syringe (available in 0.25, 0.50, 1.0 and 2.0 ml sizes) in which the solid glass plunger has been broken at some predetermined point, and (b) a syringe cap made by cutting the needle portion from a hypodermic needle and sealing the opening with solder. The syringe and both portions of the broken plunger are well lubricated with mineral oil and a few drops of mineral oil are placed in the syringe cap. The syringe is rinsed with a few drops of heparin solution, and the excess is ejected. This leaves the lumen of the needle filled with mineral oil and heparin, and displaces the last trace of air. Though the plunger of the syringe is broken, if well lubricated with mineral oil, the plunger will perform as a unit even under reasonable negative pressures. As the volume of blood drawn into the syringe reaches the desired capacity (and the break in the syringe plunger comes to the end of the barrel) the outer end of the plunger comes off easily and is removed. It is usually found that the blood pressure of the animal will continue to force the inner portion of the barrel out slightly. After the sample of blood is drawn, the needle is removed and is quickly replaced by the oil-filled syringe cap. This unit is then placed in a lusteroid centrifuge tube containing a few milliliters of mercury, is surrounded with finely cracked ice, and is centrifuged at low speeds (500-700 g for 15 minutes). At higher speeds some of the blood sample is frequently lost by leakage. If traces of mercury are found between the barrel and plunger (which will cause the plunger to move with difficulty) a small amount of vaseline should be spread over the
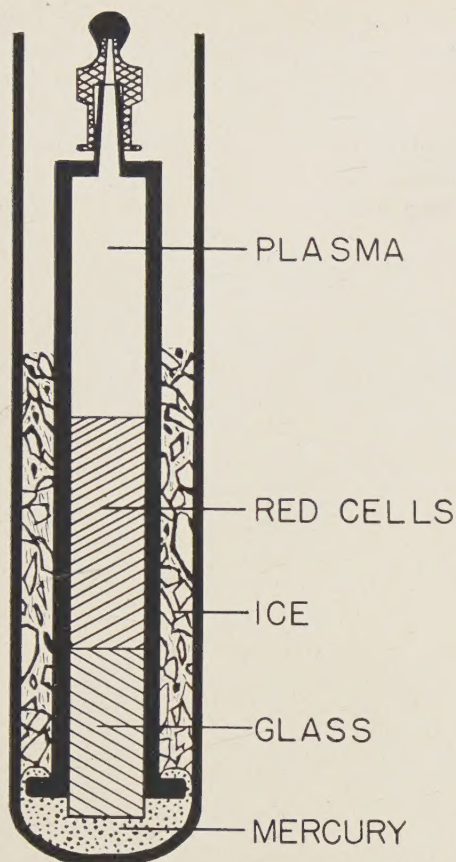
PLASMA

RED CELLS

ICE

GLASS

MERCURY

FIG. 1. Apparatus as it appears immediately following centrifugation.

lower portion of the glass unit before centrifuging, or the volume of mercury in the centrifuge tube should be decreased.

Following centrifugation it will be found (Fig. 1) that the red cells have been centrifuged into a compact layer just above the plunger of the syringe. If only minimal amounts of mineral oil have been used, the mineral oil will be found in the small tip of the syringe where it can easily be ejected. The remainder of the plunger is returned to its normal position and the samples are delivered into pipets.

With Krogh-Keys pipets(1) and the usual volumetric pipets this is easily accomplished by connecting the pipet to the syringe tip with a 1 cm length of small latex tubing, and filling the pipet with positive pressure from the syringe. With Scholander-Roughton blood pipets(2) it has been found convenient to grind the syringe tip so that the pipet tip fits into it. Between aliquots the cap was returned to the syringe tip.

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Glomerular Intermittence in Rats Demonstrated by Use of a New Direct Visual Technic. (19947)

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Incidental to a broader study of the renal vascular bed in various physiological and pathological states of function, we have gathered material which seems clearly to demonstrate glomerular intermittence in the rat kidney.

Glomerular intermittence was first observed in the frog by Richards *et al.*(1) and its occurrence in amphibia is widely accepted. However, the belief that "all glomeruli function all the time" in mammals has been

widely held. Smith(2) and others believe their work utilizing clearance technics constitutes firm proof of this statement if not in all mammals at least in the dog and humans. The work of Kaplan and Smith(3) using clearance technics has demonstrated glomerular intermittence in rabbits. This work has been both confirmed and disputed by others.

Methods. Our technic consisted in the following: 1) At a preliminary operation (under ether anesthesia) rats were opened, the abdom-

inal aorta visualized, and a clamp placed on it a few mm above its bifurcation. Using a straight cutting edged needle, a small opening was made in the aorta just above the bifurcation and a No. 10 polyethylene tube (previously filled with normal saline) was inserted into the opening. The clamp was then removed from the aorta and the tubing was pushed craniad so it would terminate a few (5 to 10) mm above the renal arteries. The tubing was secured by ligatures to the muscles of the posterior belly wall. The other end of the tube was brought out of the lower end of the abdominal incision and by means of blunt dissection was carried beneath the skin to a point on the back behind the head where a small incision was made and the free end of the tube exteriorized. Before closing the tube, we would demonstrate that we were in the aorta by observing a back flow of blood under pressure in the tube. The tube was then cleared with normal saline and sealed. The abdomen was then closed and the animals were allowed to recover in individual cages for not less than 2 and usually 3 to 5 days. (Animals which did not appear fully recovered after 2 or 3 days were discarded). 2) After the 2 to 5 day recovery period, various agents (2% saline, 5% urea, distilled water, pitressin, and epinephrine) intended to modify the function of the kidneys were introduced through the tubing. Dehydration for 48 hours was also used as an experimental agent. At appropriate time intervals, 1 ml India ink was rapidly (5 to 10 sec.) injected through the tubing and while the injection was proceeding the animal was guillotined at a point between the kidneys and the heart. In this manner we believe we introduced ink into the kidneys at normal pressure over the pathways which were functioning during the 5- to 10-second injection period and that by guillotining the animal, as we did, we stopped, at that moment, all movement of blood in the kidneys and therefore had an India ink outline of the vascular bed which was operative during the brief 5- to 10-second interval. The kidneys were immediately removed, fixed in 10% formalin and sections prepared for histologic study.

The technic used in these experiments is, we believe, superior to most previous experimental work of a similar nature because: (a) It requires no anesthetic agent which suppresses urine formation. (b) It requires no surgery which might disturb the nerve supply to the kidney. (c) The India ink which at the termination of the experiments
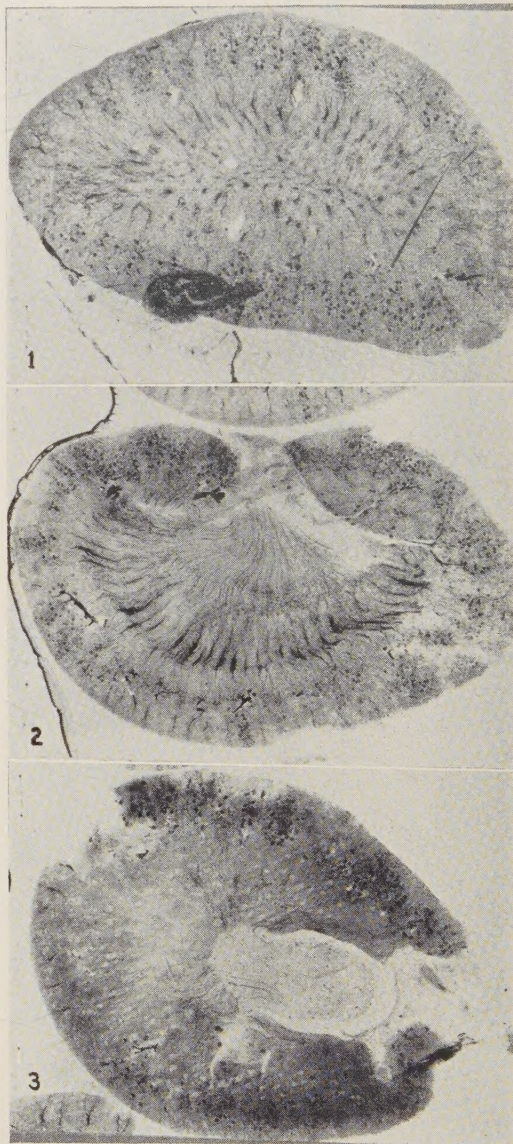

FIG. 1. Rat received standard chow and tap water until 2 hr before sacrifice when water was removed.

FIG. 2. Rat received 2% saline (1 cc/100 g) 30 min. before being sacrificed.

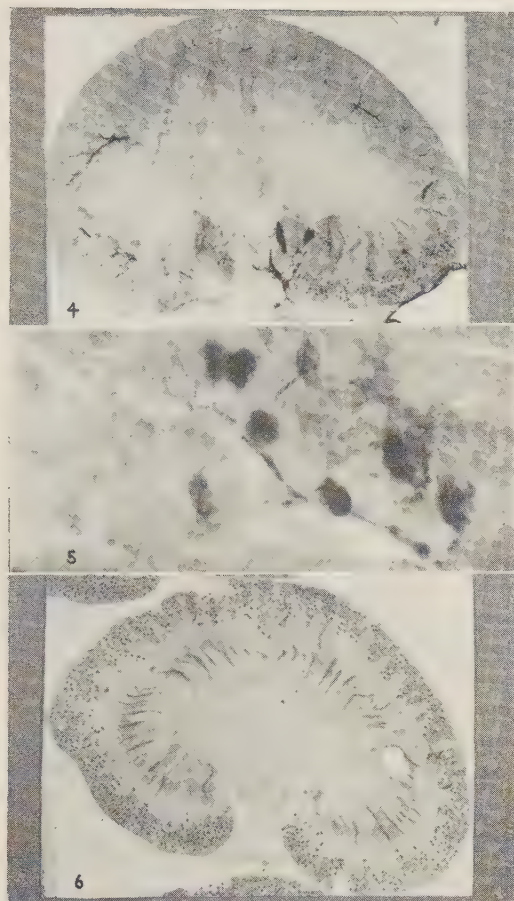FIG. 3. Rat received .2 U pitressin in ½ cc saline followed immediately (in about 5 sec.) by sacrifice procedure.

FIG. 4. Rat received ½ cc of 1/250000 epinephrine followed immediately (in about 5 sec.) by sacrifice procedure.

FIG. 5. Enlarged microphotograph of a portion of section shown in Fig. 3.

FIG. 6. Rat received no water for 48 hr prior to sacrifice.

Results. As previously stated, this is a report of an incidental finding made during the course of a larger study not yet fully completed. Of the 20 kidneys thus far studied, 9 show what we interpret as clear cut proof of glomerular intermittence.

These results were obtained under 4 different conditions: (a) One of our normals which had had the water removed from the cage 2 hours before sacrifice (Fig. 1). (b) Three animals receiving 2% saline (1 cc/100 g) Fig. 2 is typical of these. (c) Two animals receiving Pitressin (one received 0.2 U and one received 1.0 U) Fig. 3 is typical of these. (d) Three animals receiving epinephrine in 3 different dosages as follows: 1 cc—1/250000, ½ cc—1/250000, ½ cc—1/25000. Fig. 4 is typical of these.

With greater magnification (about 20$\times$), each of these kidneys shows areas such as are seen in Fig. 5 in which some glomeruli filled with ink and other "empty" glomeruli are clearly visible. The kidneys seen in Fig. 1 to 5, which we believe constitute a demonstration of intermittence, may be contrasted with Fig. 6 which is typical of the 11 kidneys in which all glomeruli were functioning at the time ink was injected.

Comments and conclusion. In analyzing our results two principal questions arose: 1) Was the ink being thoroughly mixed with blood? We believe this technic, in which the ink is injected into the aorta in a direction counter to the blood flow, would set up such an eddy as to insure very adequate mixing. (We believe the ratio of ink to blood at the point of injection is in the range of 1 to 5). The fact that all glomeruli of 11 kidneys contain ink supports this belief. Furthermore, in the 9 kidneys which show intermittence, most of the large arteries (arcuate and interlobular) contain ink. 2) Could "the empty" glomeruli have contained ink which had flowed on to the venous side? Except in a few instances we succeeded in guillotining our animals *while* the ink was being injected and we believe this stopped all pressure behind the ink column. Furthermore, India ink contains a high percentage of particles too large to pass through capillaries. In addition, the glomeruli which contain ink in those kidneys

is introduced into the kidneys, reaches the kidneys under a pressure which must be approximately the normal for the rat being sacrificed. (Most injection studies have been done on post-mortem material often at very high pressures.) Many of those in which the ink was introduced by the animal's own heart activity involved some very unphysiologic procedure such as opening the chest in order to introduce a needle into the aorta(4), etc. Most of the previous studies of a similar nature have been open to criticism on one or more of the above grounds, and we believe the technic described above successfully avoids these objections.

showing intermittence are well filled.

For these reasons, we *conclude* that no blood (and ink) was flowing through the "empty" glomeruli during the time (5 to 10 sec.) while the injection of ink was being made. In other words, the "empty" glomeruli were not functioning or more precisely blood was not flowing through them as it was in those glomeruli which contain the ink.

Attention is drawn to the fact that the functioning glomeruli in the 9 kidneys showing intermittence seem to be predominantly though not exclusively of the juxta-medullary type.

Summary. A new direct visual technic for studying the vascular supply of the rat kidney is described. Photographs of kidneys which demonstrate glomerular intermittence are presented.

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Effect of Estrogens on Serum Precipitable Iodine.* (19948)

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It is known that the serum precipitable iodine (SPI), a reliable index of the level of the circulating thyroid hormone, increases during normal pregnancy(1). The rise occurs early in pregnancy, is maintained until after delivery, and is not associated with clinical hyperthyroidism(2). The cause of the increase in the level of the circulating thyroid hormone has not been explained.

Some instances of abortion or threatened abortion in early pregnancy have been reported to be associated with levels of the SPI below that found in normal pregnancy and the administration of exogenous thyroid has been advocated in these cases(1,2). On the other hand the Smiths(3) and others have suggested the use of estrogens in cases of habitual abortion. In other studies the pregnant state appeared partially to ameliorate clinical hyperthyroidism(4). Because of these observations and because estrogens appear to depress the respiration of tissue homogenates(5) it seemed pertinent to investigate the influence of estrogens on the SPI.

Materials and methods. Sixteen patients (5 women and 11 men) whose ages ranged from 18 to 74 years were given either diethylstilbestrol† (14 patients) or Premarin† (2 patients). None was acutely ill although the majority were chronically ill with carcinoma of the breast or prostate. From 20 to 100 mg of diethylstilbestrol per day and 5 mg of Premarin per day were administered in divided doses. The SPI was determined by the method of Barker(6) as modified by Danowski and associates(7). Our normal range, obtained on 63 individuals, varies from 3.5 to 7.3 μg per 100 ml of blood (mean 5.2 μg) and is
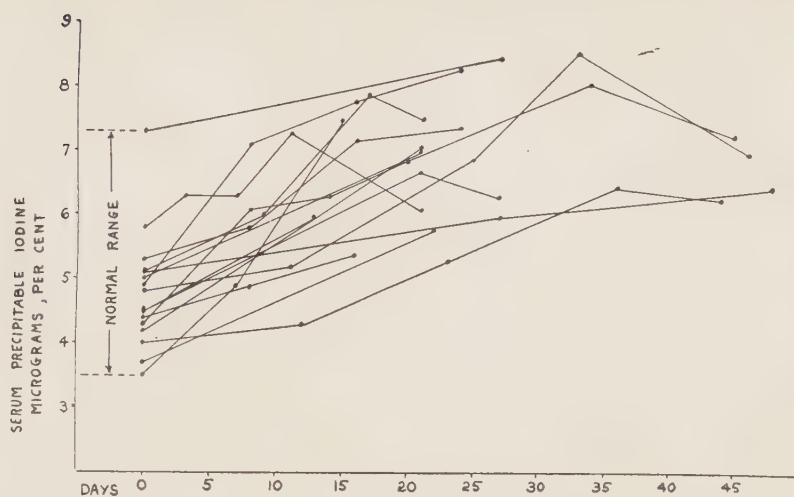
FIG. 1. Response of serum precipitable iodine (SPI) to administered estrogen; estrogen was begun on day No. 1.
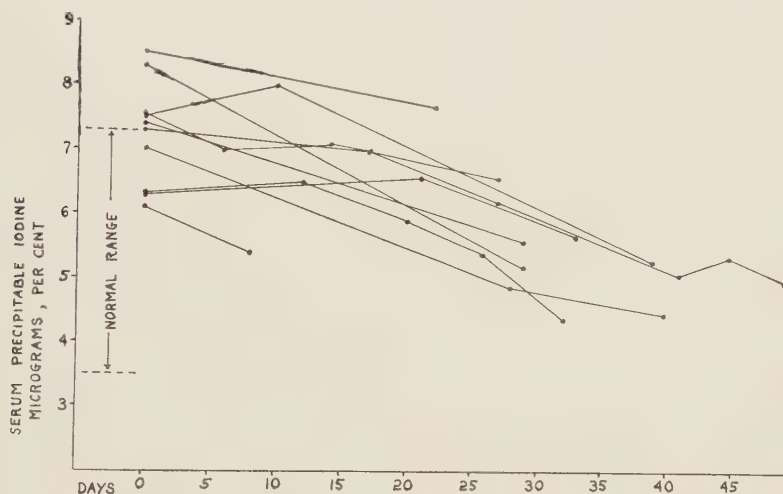

FIG. 2. Response of serum precipitable iodine (SPI) when previous therapy with estrogen was discontinued.

practically identical with that reported by others(8). In our experience and others(7,8) the SPI remains relatively constant in the same individual from time to time; variations greater than 1.0 microgram are unusual.

Results and comment. The data in Fig. 1 demonstrate that the SPI rose in all instances when men or women were under the influence of large amounts of estrogen. The minimal amount of estrogen required to elicit a significant response or the amount of estrogen necessary to cause a maximal rise in SPI could not be determined from these studies. In those instances where the administration of

estrogen was continued beyond two weeks a "leveling off" in the increments was usually noted by the third or fourth week. None of the patients exhibited evidences of hyperthyroidism although in several instances the SPI was clearly in the hyperthyroid range. That the rise in the SPI is probably maintained with continued administration of estrogen is suggested by the finding of a concentration of 10 μg % in a woman (not included in the figures) who had been on continuous therapy with stilbestrol for 3 months. She was clinically euthyroid and had a normal basal metabolic rate.

To substantiate further the observation that the SPI is increased by estrogen, therapy was discontinued in 10 individuals and the course of the SPI followed for varying intervals thereafter. In all instances the SPI gradually fell toward the normal control range (Fig. 2). Although the data are limited it appeared that at least four weeks were required for maximal fall.

These findings suggest the possibility that the physiologic rise of the SPI of normal pregnancy is a phenomenon secondary to increased elaboration of estrogen since early and continued elaboration of large amounts of estrogen characterizes normal pregnancy. The data may also indicate that the tolerance for thyroid hormone is increased by estrogen. The clinical implications of these findings and the elucidation of the mechanisms involved in the response, remain to be evaluated.

Summary. The serum precipitable iodine (SPI), an index of the level of the circulating thyroid hormone, rises in men or women during the administration of estrogen and falls to control values when the estrogen is discontinued.

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Quantitative Study of Arthus Reaction and of Cutaneous Anaphylaxis Induced Passively in the Rat.*   (19949)

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The severity of the Arthus reaction in relation to the amount of antibody has been thoroughly studied by Fischel and Kabat in the rabbit(1), as well as by Benacerraf and Kabat in the guinea pig(2). No information is available, however, concerning the amounts of antibody required to induce the Arthus phenomenon in the rat. Kenton(3) was able to provoke direct Arthus reactions in the rat by the intraperitoneal injection of a large amount (5 ml) of rabbit antiovalbumin serum followed 3 hours later by the intracutaneous injection of the specific antigen. The antiserum was assayed by the collodion particle method and had a titer of 1:5000, but was not analysed for its antibody nitrogen content. The present investigation was undertaken to correlate the severity of passive direct or reverse Arthus reactions in the rat with the amounts of antibody required for their production. It also deals with the production of passive cutaneous anaphylaxis, as revealed by the intravenous injection of a mixture of antigen and a dye ("Geigy Blue 536") in animals previously sensitized by the intradermal injection of antibody. Circular blue spots develop at the sensitized sites as a consequence of the release of histamine which results from the interaction of antigen and cell-fixed antibody. As shown in this laboratory(4), the latter reaction is fundamentally different in mechanism from the Arthus phenomenon.

Materials and methods. Rabbit antiovalbumin and antipneumococcus serum type III were analyzed for antibody nitrogen by the quantitative precipitin method(5). Three times recrystallized egg albumin (Ea) and a highly purified preparation of SIII were used as antigens. The anti-Ea serum contained 0.307 mg and anti-SIII serum 1.593 mg AbN per ml.‡ Dilutions of the antigens or of the antisera to be injected intracutaneously were made in saline such that desired amounts were contained in a volume of 0.1 ml. For the intravenous injections the volume was 1 ml. Rats weighing 100 ± 20 g were used throughout the experiment. The hair of the abdomen on the inoculation sites was simply clipped, no use being made of any depilatory or shaving in order to prevent nonspecific irritation of the skin. In performing the intradermal injections for the induction of passive cutaneous anaphylactic reactions care was taken not to pinch the skin and a sharp 26-gauge needle was first introduced through the skin and then had its direction reversed so as to deposit the inoculum properly in the dermal layers.

For the production of direct Arthus reactions antibody was given intravenously followed immediately thereafter by antigen intradermally. Vice versa, for the reverse Arthus reaction antigen was given intravenously and antibody intradermally. The rats were killed by hitting them on the head 4 hours later, their skin opened and reflected in order to observe the results on its inner surface. The intensity of the Arthus reactions was graded according to the diameter of the hemorrhagic area: $\pm$ (less than 5 mm), $+$ (5-10 mm) $++$ (10-15 mm) $+++$ (15-20 mm) and $++++$ (more than 20 mm). As to the passive anaphylactic reactions they were elicited by the intradermal injection of the antibody followed 4 hours later by the intravenous injection of a mixture of 0.5 ml of an adequate dilution of antigen plus 0.5 ml of 0.5% "Geigy Blue 536". The animals were

‡ We are indebted to Dr. Michael Heidelberger, of the College of Physicians and Surgeons, Columbia University, New York, for samples of type III antipneumococcal serum and type III polysaccharide (lot 202).

TABLE I. Severity of Reverse Arthus Reaction in the Rat in Relation to Amount of Rabbit Antiovalbumin.

Antiovalbumin N., µg	Reactions			
60	++++	+++	+++	+++
30	++	++	++	++
20	++	++	++	++
10	+	+	±	±
5	±	±	0	0
1	0	0	0	0

Animals received 30 mg Ea intrav. followed immediately thereafter by the intradermal dose of antibody.

TABLE II. Relationship between Severity of Reverse Arthus Reaction in the Rat and Amount of Intradermally Injected Rabbit Anti-SIII.

Anti-SIII N., µg	Reactions			
20	+++	++	++	++
10	++	++	+	
5	±	±	±	0

Animals received .25 mg SIII intrav. followed immediately thereafter by intradermal dose of antibody.

TABLE III. Passive Cutaneous Anaphylaxis in the Rat in Relation to Amount of Rabbit Antialbumin and of Rabbit Anti-SIII.

Antibody-N., µg	Reactions				
	(A) Anti-Ea				
2	++++*	+++			
1	++	++	±	±	±
.5	+	+	±	±	0
.25	0	0	0	0	0
	(B) Anti-SIII				
2	+++	+++			
1	+	+	+		
.5	0	0	0		
.25	0	0	0		

Animals received 15 mg Ea or .15 mg SIII intrav. 4 hr after intradermal dose of antibody.
* The results in the vertical columns correspond to dilutions of antibody tested on the same animal.

killed 10-15 min. after the injection of the antigen, the reactions examined and the sizes of the spots graded as described for the Arthus reactions.

Results and discussion. The results concerning the reverse Arthus reaction are summarized in Tables I and II. The data show that with both systems Ea-anti Ea and SIII-anti SIII the amount of antibody required for minimal Arthus reactions ($\pm$ to $+$) is between 5 and 10 µg AbN. This value

is of the same order of magnitude as the one found by Benacerraf and Kabat(2) for the reverse Arthus reaction with anti-Ea in guinea pigs of 250 ± 50 g.

By intravenous sensitization with anti-SIII followed by the intradermal injection of 0.20 mg SIII, direct Arthus reactions of medium severity (++ to +++) were obtained with 800 μg, + reactions with 400 μg and ± reactions with 200 μg AbN. For the minimal observable reaction (±) the amount of antibody N required per gram of rat corresponded therefore to approximately 2 μg, while for the guinea pig(2) only 0.4 μg are required per unit weight.

In the passive cutaneous anaphylaxis experiments a value of 0.5-1.0 μg AbN was found for the threshold of the reaction with both the SIII-anti SIII and Ea-anti Ea systems, in agreement with earlier findings(6). The ratios between the amounts of antibody required for minimal reverse Arthus reactions and for passive cutaneous anaphylaxis in the rat and in the guinea pig were respectively 10 and 1000-2000. Since the skin-sensitivity to histamine is about the same in the two species, it is suggested that the higher amounts of antibody required for passive cutaneous anaphylaxis in the rat be ascribed to a lesser ability of the rabbit antibody to become fixed to the dermal tissue.

The enormous difference in the minimal amounts of antibody required for passive cutaneous anaphylactic reactions, as contrasted with the essentially identical values found for the passive reverse Arthus reaction in the rat and in the guinea pig indicates once again that the two reactions are basically different in their mechanisms.

Summary. 1. The severity of the Arthus reaction in the rat was studied using known amounts of antigen and antibody. 2. Reverse Arthus reactions of minimal severity were obtained by intradermal sensitization with 5-10 μg anti-Ea or anti-SIII. 3. By intravenous sensitization direct Arthus reactions of medium severity were obtained with 800 μg and minimal reactions with 200 μg anti-SIII. 4. The threshold of the reaction in passive cutaneous anaphylactic experiments corresponded to 0.5-1.0 μg AbN for both the Ea-anti-Ea and SIII-anti-SIII systems. 5. The meaning of these findings is briefly discussed.

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Nephrotoxic Globulin Nephritis. III. Prompt Death after Administration of Nephrotoxic Globulin to the Rat.* (19950)

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Some recent studies from this laboratory have concerned the pathogenesis of nephritis produced in the rat by administration of rabbit anti-rat-kidney gamma globulin (nephrotoxic globulin, NTG)(1,2). It was

noted from the start of these studies that NTG administration produced extrarenal effects, such as intravascular hemolysis. The latter could not be eliminated completely by absorption of the NTG with homologous or heterologous erythrocytes. A variable proportion of the animals in each experiment died within the first 12 to 24 hours after NTG administration and, at autopsy, their kidneys

TABLE I. Schedule of Groups and Procedures.

Group	Title	No. animals	Sex	Material inj.	Additional procedures
1	GC-nephrectomy	10	♂	GC	Nephrectomy
2	NTG	10	♂	NTG	
3	NTG-nephrectomy	10	♂	NTG	Nephrectomy
4	NTG-cortisone	10	♂	NTG	Cortisone admin.
5	NTG-unabsorbed	10	♂	NTG-unabsorbed	
6	NTG-PBZ	4	♂	NTG	Tripelennamine admin.
7	NTG-unabsorbed-PBZ	6	♂	NTG-unabsorbed	” ”
8	NTG-female	10	♀	NTG	
9	NTG-female-PBZ	9	♀	NTG	” ”

appeared to be normal on gross examination. It had been known from previous experiments that rats of the same colony will live approximately 48 hours after bilateral nephrectomy if maintained on stock diet. In view of the complex of antigens used for the production of NTG (whole rat kidney), it seemed most likely that the prompt deaths after NTG administration were the result of extrarenal factors, although other less probable explanations could also be advanced. The present experiments were designed to separate the renal and extrarenal effects of NTG administration so that their separate relation to prompt death could be evaluated.

It may be observed in passing that there has been a tendency in the current literature for nephritis produced by nephrotoxic serum administration to be considered as the equivalent of human glomerular nephritis or lipoid nephrosis(3,4). This tendency is not adequately justified. The pathogenesis and etiology of human nephritis and nephrosis are unknown, although it is generally believed that the diseases involve mechanisms of hypersensitivity. There are readily observed differences between human nephritis, nephrosis, and nephrotoxic globulin nephritis in the rat. Among these differences are the paucity of hematuria, lipoid deposition in the kidney, and periodic acid-Schiff reagent stainable material in the rat. For the sake of clarity, it would seem desirable to consider nephrotoxic globulin nephritis and the human diseases mentioned as independent entities which are not necessarily related to one another.

Methods and materials. In this experiment 60 male and 19 female rats of the Slonaker-Addis strain were used. The animals were selected to weigh 150 g within a small range of variation. The stock diet for this colony, which contains 17% protein, was used before and during the experiment, with water *ad libitum.* The preparation of nephrotoxic globulin (NTG)† by immunization of rabbits against a suspension of whole rat kidney, and the preparation of control rabbit globulin (GC) previously has been described in complete detail(2). In order to eliminate the influence of variable potency, observed in NTG preparations, all these experiments were performed with a single lot (8-9-51). Some experiments were performed with NTG which had not been subjected to absorption with erythrocytes to remove anti-rat-erythrocyte antibody (NTG-unabsorbed). NTG, NTG-unabsorbed, and GC (Lot 3-3-52) were administered by injection in a foot vein, in equivalent amounts: 23.0 mg protein in 1.0 ml 0.85% sodium chloride solution. Bilateral nephrectomy was performed under light ether anesthesia, before injection of the globulin. The entire procedure, which utilized the transperitoneal approach through a flank incision on each side, required about 7 minutes for each animal. Muscle and peritoneum were closed in one layer with silk, while the skin was closed with Michel clips. After the experimental manipulations, the animals were returned to the cages and were inspected at intervals to ascertain the number of survivors. After 50 hours from the beginning of the experiment the remaining animals were discarded and the experiment was concluded.
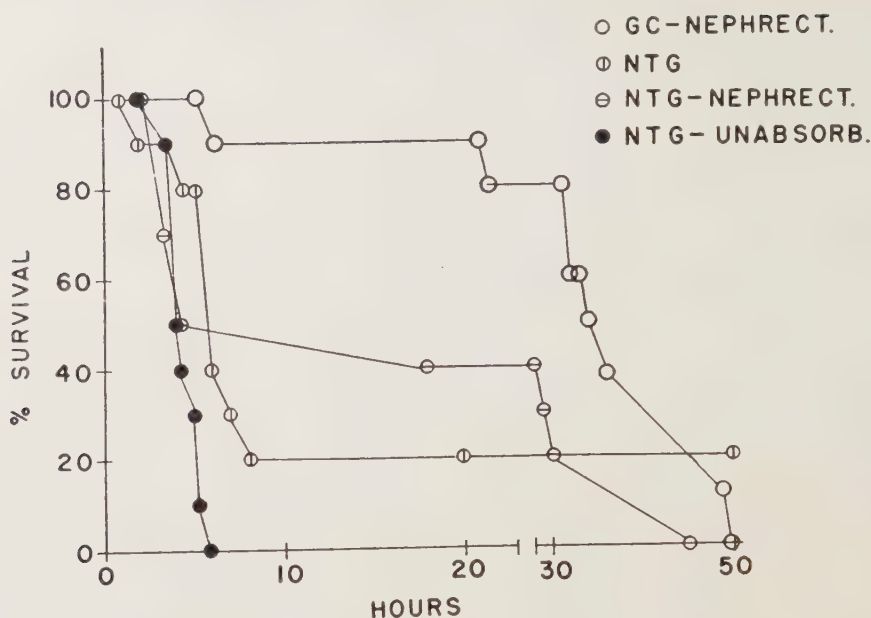
The various groups and experimental con-

FIG. 1. Survival of rats after nephrectomy and after injections of NTG and GC.

ditions for each are noted in Table I. In group 4 cortisone acetate‡ (2.5 mg in 0.1 ml) was administered by intraperitoneal injection 30 minutes before NTG administration. In groups 6 and 7 tripelennamine hydrochloride§ (1.0 mg in 0.2 ml) was administered by intraperitoneal injection 30 minutes before NTG administration. In group 9 the same dose of tripelennamine was repeated for a total of 3 doses at intervals of 2 hours.

Results and discussion. The curve for group 1 (Fig. 1) indicates the mortality from bilateral nephrectomy in the rat, under the conditions used for these experiments. Most of the animals died between 22 and 36 hours after operation and the remaining ones succumbed before the 50th hour.

For the purposes of this discussion we can consider the NTG preparation to contain 3 classes of antibodies: anti-rat-kidney, anti-rat-erythrocyte, and anti-rat-tissues. Anti-rat-kidney is intended to include only antibodies formed to specific kidney proteins from renal parenchymal cells. Anti-rat-tissues

‡ Cortone acetate, kindly provided by Dr. Leo V. Curtin, Merck and Co., Rahway, N. J.

§ Pyribenzamine hydrochloride, kindly provided by Dr. Ernst Oppenheimer, Ciba Pharmaceutical Products, Summit, N. J.

is intended to include all other antibodies (excluding anti-rat-kidney and anti-rat-erythrocyte) formed in response to serum protein, connective tissue, nervous tissue, fat, and other elements which are contained in a whole kidney suspension but are not specific to it. In Fig. 1 it can be seen that the injection of NTG-unabsorbed, which contains all the 3 classses of antibodies defined above, lowers the survival curve in a striking manner, so that all of the animals died within 6 hours of the injection. From this it is clear that the prompt death after injection of NTG-unabsorbed cannot be explained even by the assumption that there has been total cessation of renal function.

Not all the animals died when NTG (which was absorbed with rat erythrocytes) was administered. The survival time was somewhat extended, although still very significantly lower than the survival time after bilateral nephrectomy. This led to the conclusion that, while a definite fraction of the prompt lethal effect resides in the anti-rat-erythrocyte antibody, the principal effect is associated with the other 2 antibody classes. However, administration of NTG after nephrectomy yields a survival curve nearly identical with that obtained after NTG administration in the
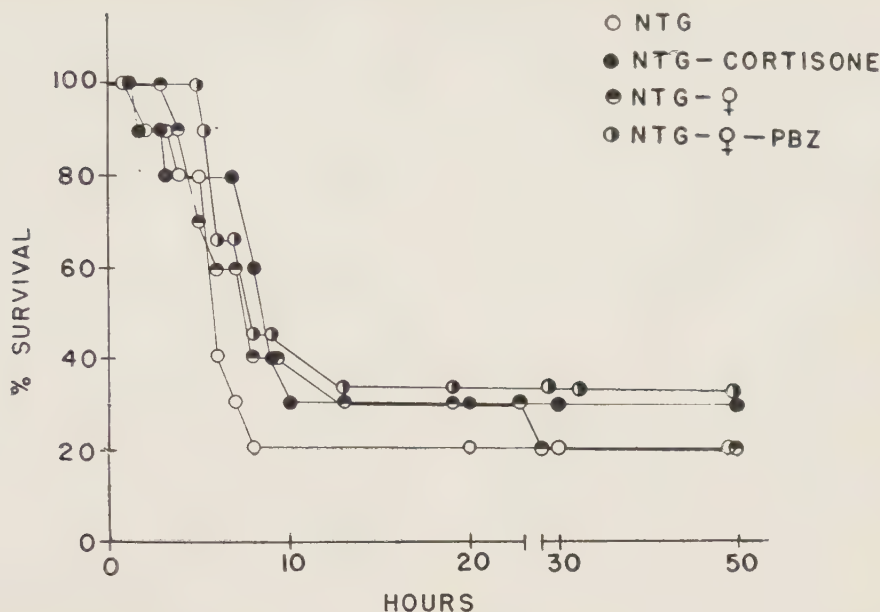
FIG. 2. Survival of rats after injection of NTG; influence of cortisone and tripelennamine on survival.

intact rat. The latter observation eliminates the possibility that the prompt lethal effect is the result of interaction between anti-rat-kidney antibody and kidney tissue. Therefore, the final conclusion is inescapable that the principal prompt lethal effect of NTG must reside in the anti-rat-tissue antibodies, with an additional effect contributed by the anti-rat-erythrocyte antibody.

The effect of NTG administration was studied after administration of cortisone and tripelennamine. In Fig. 2 it can be seen that neither cortisone nor tripelennamine significantly affected the survival curve. Experiments with NTG-unabsorbed and tripelennamine gave a similar result. It can also be seen that there is not a significant sex difference in the survival curves (Fig. 2, NTG and NTG-female), contrary to the sex difference observed in manifestations of NTG nephritis after 4 weeks have elapsed(5).

It has been shown by Pressman and his associates(6,7) that anti-rat-kidney serum prepared by the same method as NTG but unfractionated and unabsorbed with erythrocytes, will localize in a variety of organs and tissues. These include kidney, liver, lung, spleen, blood and, to a lesser degree, brain and heart. Previous work has also shown a toxic effect of NTG on tissue culture explants of kidney, heart, and brain(8). Such results may reasonably be explained by the complex antigen composition of ground, whole kidney suspension, previously mentioned. The minor constituents of kidney tissue may be expected to become more significant antigens during the prolonged series of injections required to produce sera of high titer, and this circumstance will increase the occurrence of significant antibody titers to extrarenal tissues. In addition, several investigations have shown that multiple injections of a single purified antigen may also result in diminished specificity of the high titer antisera(9). Even those antisera produced with antigens obtained from partial tissue fractionation(10,11) must have a complex composition. The glomerular antigen of Greenspon and Krakower(11) contained "minimal non-glomerular contaminants" as well as soluble material liberated during the fractionation process from crushed cells. Glomeruli themselves contain endothelium, epithelium, basement membrane, and other tissue constituents, and consequently may contain multiple antigens, not necessarily peculiar to the kidney.

These considerations support the proposition that nephritis produced by NTG administration cannot be compared directly with human glomerular nephritis and lipoid nephrosis; the possibility that a study of NTG nephritis may lead to generalizations which are relevant to the study of human nephropathies cannot be denied. Further, the prompt deaths that follow NTG administration in the rat cannot be considered to result from the nephrotoxic action of NTG, but are the result of extrarenal effects.

Summary. Administration of nephrotoxic globulin (NTG) to the rat produces death in less than 12 hours if the amount given is sufficient. Bilateral nephrectomy usually does not lead to death in less than 24 hours, when the animals are maintained on a stock diet containing 17% protein. The prompt death is related principally to anti-rat-tissue antibodies other than anti-rat-kidney. Anti-rat-erythrocyte is of definite but minor significance in the production of prompt death. Cortisone and tripelennamine exert no protection against the prompt death described. It is evident that nephrotoxic globulin (NTG)

has important extrarenal effects which must be considered in comparing NTG nephritis with other similar diseases of the kidney, such as those of natural occurrence in man.

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The Chemical Nature of Encephalitogenic Proteolipide A and B. (19951)

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The brain proteolipide A and B (PL, AB), a new type of tissue lipoprotein recently discovered by Folch and Lees(1), deriving from the mouse, was found to be capable of inducing acute disseminated encephalomyelitis in mice indistinguishable from that brought about by the injection of whole brain(2,3). It was also shown that PL, AB possessed a degree of encephalitogenic power similar to that of whole brain and that the latter, after this constituent had been extracted, was inactive. It was therefore assumed that all of the encephalitogenic agent was present in the PL, AB fraction. An attempt was then made to

test for activity the remaining fractions of the extract; to break up the PL into smaller units and to determine whether the active agent could be identified with any one of these components. The present paper describes these investigations.

Materials and methods. The PL, AB was prepared from mouse brain and the H-line W-Swiss mice(4) were employed as experimental animals. All fractions were mixed with the Freund-type adjuvant and inoculated into mice subcutaneously, 6 times or less, at weekly intervals. The methods of preparation, inoculation and observation of reactions in animals have already been described(2-5). Proteolipide C (PL, C) was obtained following closely the technic of Folch and Lees(1):
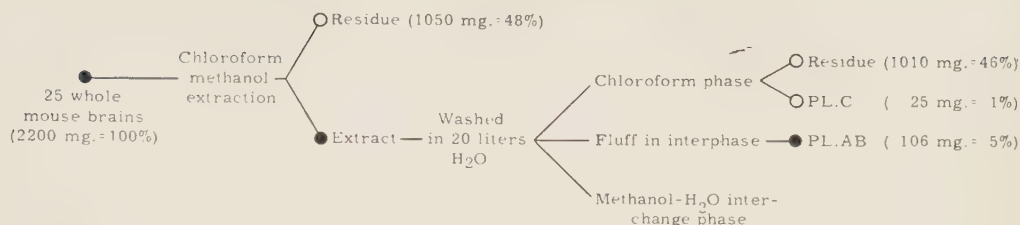
FIG. 1. Encephalitogenic activity of various fractions of mouse brain. ○ = negative; ● = positive reaction of experimental encephalitis in mice; PL = proteolipide; mg = dry wt.

the yield from 53 g dry (240 g wet) weight, mouse brain was 600 mg or about 1%. PL, C supernates were collected, pooled and concentrated by drying in a vacuum (residue of chloroform phase in Fig. 1). Fractionation of PL, AB into lipid and protein moieties followed the method of Folch and Lees(1,6): 60 g (150 fresh mouse brains) yielded 636 mg dry weight of fluff which contains PL, AB. The frozen fluff, collected on filter paper, was dried for 6 hours in a $CaCl_2$ desiccator *in vacuo* and then stored at –10°C overnight. To the material resuspended in 6 ml distilled water in a suction flask, 100 ml chloroform were added. Methanol was added to the resulting milky suspension until one phase was obtained then the solvent was removed by vacuum distillation. 100 ml of hot chloroform-methanol (2:1 by volume) were added and the precipitate filtered through fat-free filter paper. The extraction was repeated and the yield of precipitated protein was 200 mg, about 31%. The supernate was evaporated to dryness and yielded 400 mg lipid, about 63%. Attention should be called to the fact that Folch and Lees(1) found that dissolving the PL, AB in chloroform-methanol-water mixture and removing the solvents by vacuum distillation, thus drying it, splits the bond between protein and lipid and consequently separates them. After the separation the protein is rendered insoluble in the chloroform-methanol-water mixture, the amount of the insoluble protein can then be a measure of the extent of the splitting of the PL, AB complex.

Other preparations for test of their encephalitogenic activity consisted of: 1) PL, AB dried, but not extracted with hot chloroform-methanol; *i.e.*, the substance was dried over $CaCl_2$ *in vacuo* over night at room temperature. 2) This process of drying was repeated after resuspension in the solvent mixture. 3) To dried PL, AB hot chloroform-methanol (2:1 by volume) was added and the solvent removed by evaporation without separation of component protein or lipid. 4) To fresh, not dried PL, AB in water suspension hot chloroform-methanol was added; it was kept then in a water bath at 100°C until all the solvent was evaporated, the residue consisting of the treated PL, AB in a water suspension. In Fig. 1 and Tables I and II will be found a summary of the results of tests on these various preparations of PL, AB and its components.

Experimental. The fact that all of the encephalitogenic activity of brain tissue is contained in the PL, AB fraction has already been shown(2,3) and results of tests with the fractions given in Fig. 1 and Table I support this finding: of the 4 fractions of brain, obtained by extraction with chloroform-methanol and washing, only PL, AB was regularly active. That is, of 2200 mg dry weight of brain, 106 mg PL, AB were secured which represents 5% of the solids of the original brain, and all mice inoculated with this material developed encephalomyelitis. The PL, AB-free residue amounted to 1050 mg, or 48%, and was inactive; so was the 25 mg, or 1% fraction PL, C (Fig. 1). Only 1 of 34 mice in 2 tests developed the experimental affection after inoculation of the residue of the chloroform phase which weighed 1010 mg or 46% and represented substances other than PL, C soluble in the chloroform. It is not certain whether this single positive animal might not have received an inoculum contaminated slightly with PL, AB. The residue of the whole-brain; PL, AB; PL, C; and the residue of the chloroform phase (Fig. 1) accounted for 100% of the solids in the original brain tissue;

TABLE I. Encephalitogenic Activity in Mice of Fractions of Mouse Brain.

Fraction	No. of mice	Dry wt, mg/ml	Total mg dry wt in 6 inj. in 1.8 ml	No. pos./neg.	% pos.	Brain wt equiv., mg/ml	
						Wet	Dry
Whole brain (control)	20	22	39.6	20/0	100	100	22
PL. AB*	20	1.06	1.91	20/0	100	100	22
PL. C	20	6	10.8	0/20	0	2400	528
Residue of PL. C {	14	121	217.8	0/14	0	1200	264
{	20	20.2	36.36	1/19	5	200	44

* PL. AB = Proteolipide A and B.

TABLE II. Encephalitogenic Activity of Proteolipide (PL.) Fractions.

PL. fraction	No. of mice	Dry wt, mg/ml	Total mg dry wt in 6 inj.	No. pos./neg.	% pos.	Protein insol. in chloroform-methanol, mg/ml
PL. AB (control)	30	1.06	1.908	30/30	100	0
Protein	20	1.20	2.16	0/20	0	—
Lipid	20	2.09	3.762	0/20	0	—
Desiccated PL. AB	20	1.49	2.682	7/20	35	.435
Desiccation repeated PL. AB	20	2.50	4.55	0/20	0	.750
Treated,* desiccated PL. AB	40	2.50	4.55	0/40	0	.750
Treated, fresh PL. AB	20	2.50	4.55	13/20	65	.742

* Treated with hot chloroform-methanol mixture.

the only possible remaining fraction of this system was that of the upper water phase, above the fluff.

The next step was an attempt to identify the encephalitogenic activity of PL, AB with either its protein or its lipid moiety, and to note the influence in this relation of breaking, by various manipulations, the bond between the two. For Folch and Lees(1) have stated that this bond can be split simply by drying the solutions as prepared. The results are summarized in Table II: in the last column, the amount of separated protein was determined by the quantity rendered insoluble in chloroform-methanol.

It will be observed that the protein and lipid constituents, separately, were inactive whereas the whole PL, AB, was encephalitogenic. On the other hand, a single desiccation over $CaCl_2$ at room temperature for 15 hours definitely reduced the encephalitogenic power of PL, AB; such desiccation when repeated destroyed this activity completely. In the latter instance, all of the protein moiety (30% by weight) was rendered insoluble in the chloroform-methanol-water. In another experiment shown in Table II, treatment of

the fresh, not dried PL, AB with hot chloroform-methanol followed by evaporation of the solvent, a procedure which should not disturb the bond between its protein and lipid constituents, caused destruction of 35% of its encephalitogenic power. This treatment plus desiccation, however, produced a complete loss of activity of the proteolipide.

Discussion and Summary. An explanation of the difference in results obtained by treating PL, AB with hot chloroform-methanol, and with this solvent plus drying, may lie in the fact that during the treatment a certain amount of PL, AB at the surface desiccates at the same time that the solvent evaporates. Thus the loss of activity caused by partial drying corresponds with the surface area as well as with the original concentration of brain tissue, *i.e.*, with the amount of PL, AB exposed to desiccation. For example, a suspension of PL, AB treated the same way except that a container (wide flask) permitting much wider surface was employed, became completely inactive; whereas the same suspension in a container (test tube) having only a small surface exposed, revealed a loss of 35% of its activity.

The various fractions deriving from the extract of mouse brain secured during the procedure of isolation of PL, AB were tested in mice for their encephalitogenic activity. It was found that the PL, AB, which amounted to 5% of the original constituent solids, was at least as active as whole brain, but the residue obtained after extraction with a chloroform-methanol-water mixture; proteolipide C; and the residue of the chloroform phase, all of which aggregated the remaining 95% of the original solids, were inactive. Thus from the procedure here employed it appeared that the encephalitogenic power of brain tissue, as revealed by inoculation of mice, was possessed only by one of its constituents, namely, PL, AB.

The encephalitogenic activity of PL, AB was evinced only if the protein and lipid constituents remained in chemical combination. If the bond was split, even by the milder procedures, the resultant material was found to be inactive, as were the separated protein and lipid fractions. The possibility remained, however, that the methods of splitting the bond used in this study might have changed the chemical composition of the constituents. Further work on fractionation by other means would therefore appear to be indicated.

Conclusions. Of 4 fractions prepared by chloroform-methanol extraction of mouse brain, only one, proteolipide A and B, is encephalitogenic in mice. Its desiccation which brings about a split in the bond between its protein and lipid constituents results in a loss of its encephalitogenic power and the separated protein and lipid fractions are also inactive.

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Partial Purification of Bovine Intestinal Alkaline Phosphatase.* (19952)

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Hers(1), and Kallman(2) reported that intestinal alkaline phosphatase is concentrated in the microsome fraction of rabbit mucosal cells. We have confirmed this observation, and found that the same is true in the intestinal mucosa of the steer. It is the purpose of this report to demonstrate that the isolation of the microsome fraction may advantageously be made the initial step in the preparation of a soluble preparation, with reproducible properties, from steer mucosa. This method avoids the use of preliminary autolysis or tryptic digestion, heretofore considered essential to the purification procedure, and seems more likely to lead eventually to the isolation of a pure enzyme.

Materials and methods. The bulk separation of steer intestinal microsomes was carried out on material obtained within 30 minutes of slaughtering, kept at 2°C, and was performed as follows: the inside of the duodenum was rinsed with 0.25 M sucrose, and the mucosa stripped from it. The mucosa was suspended in 2 volumes of 0.25 M sucrose, the pH of which had been brought to 8.0 with KHCO₃. The suspension was homogenized in a Potter-Elvehjem homogenizer designed to

handle large quantities of material. The homogenate was made up to a volume equal to 10 times the initial weight of the mucosa by the further addition of sucrose solution. It was then centrifuged at 14,000 × g for 10 minutes in the Spinco preparative ultracentrifuge. This treatment removed the cell fragments, nuclei, and mitochondria. The sediment was resuspended and brought to a volume 5 times the weight of the mucosa, and recentrifuged. The 2 supernatants, which contained the microsomes, were combined. The microsomes were brought down by adjusting the pH of the combined supernatants to 5.0 with 0.1 N acetate buffer, and centrifuging at room temperature after agglutination. This procedure was adopted after it had been found that the results were comparable to those obtained with microsomes isolated by high speed centrifugation from 0.25 M sucrose. The precipitated microsomes were washed twice with distilled water, and finally suspended in a volume of distilled water equal to one-half the original weight of the mucosa. The suspension was placed in a bath at 37°C and one-half volume of n-butanol saturated with water was added slowly with rapid stirring. The rapid stirring was continued for 30 minutes. At the end of this period, the emulsion was broken by centrifugation, and the aqueous layer removed. The butanol layer and the gel, which appeared at the interface of the water and butanol, were reextracted with one-half the original volume of water. The aqueous layers were combined, and lyophilized. Enzymatic activities were determined by the hydrolysis of p-nitrophenylphosphate, at pH 9.1 and 37°C. The buffer used was 0.1 M bicarbonate-carbonate, containing 0.001 M magnesium acetate. Protein determinations were performed with Lowry's modification of the Folin-Ciocalteau reagent (3). All protein values are referred to an albumin standard. The solubility of the soluble material obtained from the butanol treated microsomes was studied in acetone-water mixtures at 2°C. A 2% solution of the lyophilized material was made in water at 2°C. The solution was allowed to stand for several hours at this temperature, and then centrifuged. Any insoluble material was removed, and the

supernatant used for the precipitation studies. Two ml of the solution were used for each determination. Sufficient water and acetone were added to obtain the desired acetone concentration in 8.0 ml of solution. Protein concentration and enzymatic activity were determined on the supernatants after removal of the precipitated material. Filter paper electrophoresis studies were made on the 2% aqueous solution prepared for the solubility studies, and were carried out at pH 8.6 (0.05 M veronal) and pH 6.9 (0.05 M imidazole). The procedure was essentially the same as that described by Kunkel and Tiselius (4). The protein was detected by its color reaction with 0.1% ninhydrin in water-saturated butanol. A second, parallel strip, was used to determine the position of the enzyme. This strip was cut into 1 cm pieces, and the enzyme was eluted from each piece and the activity determined.

Results and discussion. In our investigation of methods for the purification of intestinal alkaline phosphatase the procedure initially followed was that of Albers and Albers (5). The crude enzyme was isolated from an autolysate of steer mucosa, and then subjected to fractional precipitation with acetone in the cold. It was thought that by suitable variation of the conditions of precipitation and extraction, the protein impurities might be removed. The results were unsatisfactory, since the impurities appeared to have solubility characteristics which were virtually identical with those of the enzyme. Filter paper electrophoresis of the various fractions at pH 8.6 yielded, in each case, a single long streak of anionic protein with no well-defined

TABLE I. Alkaline Phosphatase in Homogenate and in Soluble Fraction from Butanol Treated Microsomes.

| Preparation | Specific activity* | | Purification† |
	Homogenate	Soluble fraction from microsomes	
1	4.55	115	25
2	8	118	15
3	5.4	128	24

* Expressed as mM of p-nitrophenylphosphate split/hr/mg protein.
† Ratio of specific activity of soluble fraction from microsomes to that of the homogenate.

peaks. The enzyme presented a similar picture superimposable upon the protein.

Separation of the microsome component of the cell from the rest of the cellular material results in a 3- to 5-fold increase in the specific activity of intestinal alkaline phosphatase. Although this increase is not great, 70 to 80% of the extraneous cellular protein has been removed, leaving a system which is better defined than either an homogenate or an autolysate of mucosa.

The butanol extraction, similar to that used by Morton(6), and Zittle(7), was selected because of their success in obtaining soluble phosphatase preparations, and because it might be expected to disrupt a lipid-containing complex such as the microsome. The extraction results in a clear, aqueous solution of alkaline phosphatase which is not sedimentable by centrifuging for 2 hours at 20,000 × g. Denaturation of some of the other proteins in the microsomes during the butanol treatment probably contributes to the purification of the enzyme despite the fact that some loss of enzyme also occurs and the recovery at this step is approximately 50%. The purification achieved is variable, and seems to be inversely related to the activity of the initial homogenate, since the final activity of each of the 3 preparations studied is approximately the same (Table I).

Despite the considerable degree of purification obtained, the material from butanol-treated microsomes still contains a considerable amount of inert material. The amounts of protein and enzymatically active material precipitated at various acetone concentrations are shown in Fig. 1. Although the bulk of the enzyme is precipitated between 40 and 50% acetone, little protein precipitates until the acetone concentration is above 50%. Indeed, the amount of protein precipitated between 40 and 50% acetone was too small to be measured accurately as the experiments were carried out. From the amount precipitated above 50% acetone, it has been estimated that the amount of protein precipitating with the enzyme could not be more than 8-10% of the protein present.

The same conclusion is indicated by the electrophoretic results. Fig. 2 shows the dis-
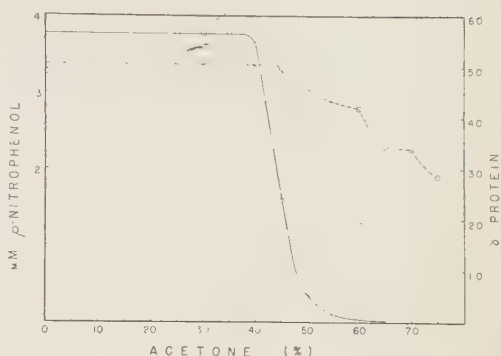
FIG. 1. Comparison of solubility of enzyme (solid line) and protein (dashed line) at increasing concentrations of acetone, in the soluble fraction of microsomes after butyl alcohol treatment. Enzyme activity at left represents mM of p-nitrophenyl phosphate split, under identical conditions, by aliquots of supernatant from the precipitation at each acetone concentration. Protein remaining in supernatant is indicated on scale at the right.
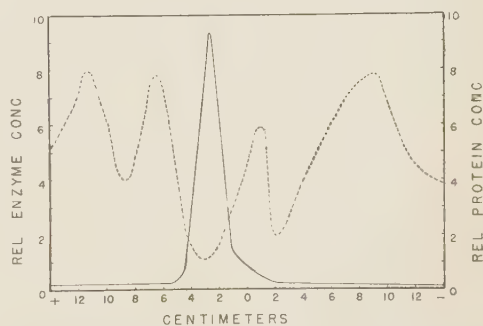
FIG. 2. Results obtained on subjecting the soluble fraction from butanol treated microsomes to electrophoresis in filter paper at pH 8.6. Distance of migration is shown at bottom. Relative concentration of protein is indicated by dashed line, the scale in arbitrary units on right. Enzymatic activity is shown by solid line, the scale in arbitrary units on left.

tribution of the enzyme after electrophoresis for 16 hours at 5.7 volt/cm, pH 8.6. The positions of several distinct protein peaks are shown, and it will be seen that the enzyme is not associated with any of these. From an evaluation of the strips developed for protein, it can be concluded that at least 90-95% of the protein is material other than alkaline phosphatase. The results obtained at pH 6.9 are similar.

Summary. A procedure has been presented for obtaining a soluble preparation of intestinal alkaline phosphatase from the microsomes of steer intestine mucosa by extraction

of the microsomes with n-butanol. Although the purification achieved is only 15 to 25 times that of the original homogenate of the tissue, the resulting preparation appears to be particularly suitable for further purification by fractional precipitation with acetone.

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Duration of Drug Action. (19953)

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A drug effect has two major features, 1) *intensity* and 2) *duration*. Intensity of drug effect is unquestionably a variable magnitude, but its quantitation meets considerable obstacles because its dimension is difficult to define. The assumption, followed in some cases, that intensity of effect can adequately be measured in such simple dimensions as length (muscle shortening), volume (glandular excretion), weight, etc., is certainly fallacious; in other cases, *e.g.*, drug-produced convulsions, one is at a loss to quantitate in any physical dimension. Yet the need to analyze the relationship between intensity of effect and its independent variable, *drug dose*, and to compare intensities of effect of different drugs has led to the elaboration of a workable yardstick for intensity of effect. This was possible by circumventing the problem of the physical dimension and focusing attention to the biological dimension. The biological yardstick is "notched" in terms of *endpoints, i.e.,* selected effects of well specified intensity, such as: *threshold effect; peak effect* = greatest intensity attained in the course of the action of a dose; *maximal effect* = greatest intensity obtainable with any dose; and numerous intermediate endpoints of appropriate specification. This instrument, refined by an abundant armamentarium of biostatistical technics to overcome variability, has placed evaluation of intensity of effect on a satisfactory conceptual and procedural basis.

The concepts and principles for quantitation of the other feature of drug effect, namely, the *duration*, are as yet on an incomparably lower level of development. This may seem paradoxical because, at first thought, the problem of dimension does not appear to offer any difficulties; by definition, the temporal features of drug effect present themselves in the physical dimension of time. Nevertheless the concept of *duration of effect* has remained rather vague and hence of little constructive value. The reasons become obvious when the intensity and time characteristics of the effect of a drug dose are represented graphically (Fig. 1). Such a diagram points out that, quite similar to the intensity axis, the temporal axis of effect is also subdivided into biological "endpoints". Since evidently the temporal features are correlated with intensity, the temporal endpoints are connotatively coupled to those of intensity. As exemplified in the diagram for action III, the temporal endpoints are time intervals measured from the time of drug administration, T_o, to that of an endpoint of intensity of effect. Major temporal endpoints are: *latency time* = time of onset of threshold effect (T_a); *peak time* = time of (onset of) peak effect (T_i); *persistence time* = time of disappearance of threshold
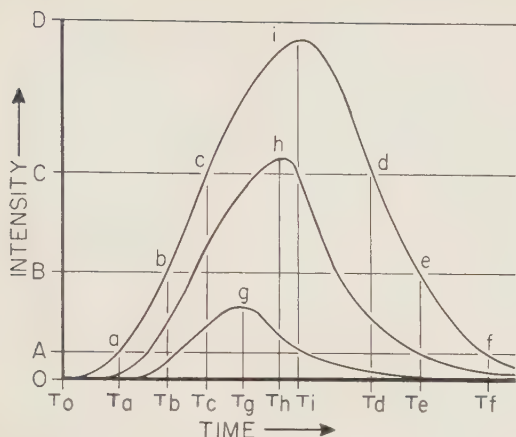
FIG. 1. Time-intensity curves of effect of 3 different drug doses, I, II and III. A: threshold effect; B: therapeutic effect; C: toxic effect; D: maximal effect. g, h, i: peak effects of the 3 doses (time sequence g<h<i selected as one out of 3 possibilities). T_g, T_h, T_i: peak times of the 3 effects. For other explanations see text.

effect (T_f). Other endpoints are the times of onset and disappearance of other intensities of effect, such as T_b, T_c, T_d, etc. It is obvious that an important use of the temporal endpoints is to measure differential periods of time, such as (T_f-T_a), (T_e-T_b), (T_d-T_c), etc., and that only these periods are "durations" (total duration of effect, duration of therapeutic or toxic effect, etc.).

These contemplations are not submitted for their academic significance; very practical necessities,—experimental, therapeutic, and toxicologic,—call for their consideration. Need and remedy may be illustrated by two examples.

1) *Duration of effect* is an important connotation of the action of drugs. Particularly in the field of short-acting antibiotics, *e.g.*, penicillin, a vast amount of work has been done to determine duration of effect and its prolongation by numerous so-called "repository" measures. Since, with secondary drawbacks, blood level of antibiotic activity can be a yardstick of intensity of effect, the time of persistence of a certain blood level of activity is usually employed to indicate duration of effect. However, the results derived from the host of investigations on repository agents are presented in surprisingly indefinite terms; duration of effect is merely expressed either by the latest time at which a thera-

peutic level of antibiotic activity was still found in the blood of an occasional individual or by the percentage of individuals still exhibiting such a blood level of antibiotic activity at a certain hour. It was in order to avoid such inadequacies that this writer has recently suggested the concept of *median persistence time* (PT_{50}) and the means of applying it for obtaining biostatistically defined values for duration of effect(1). Quantal (yes-or-no) statements on persistence of an appropriate endpoint (of intensity of effect or of antibiotic activity in the blood) are procured from a population at various, adequately narrowly spaced intervals around the expected persistence time; these statements are subjected to probit analysis, the median value (PT_{50} of the decremental endpoint effect) is established by interpolation, and the variation of sensitivity in the population is determined by an appropriate statistical procedure. (Further details will be found elsewhere)(2).

2) *Comparisons of the potency* of drugs are the pharmacologist's daily bread and the main approach to new and improved drugs. All such comparisons repose on the concept of ED_{50}. This is the dose whose *peak effect* reaches a certain intensity in 50% of experiments, and (relative) potency is the ratio: ED_{50}standard/ED_{50}test drug. Modern estimates of ED_{50} are so highly elaborate that an ED_{50} can hardly appear in public without an ornate biostatistical attire. Unfortunately, however, this attire may adorn a value which lacks the main criterion of an ED_{50}, especially in many of those cases in which the assay procedure does not automatically record the entire rise and fall of the individual effect of the test doses. The intensity is then often measured at a uniform, rigidly pre-set time interval after drug administration and no precautions are taken to insure that this time interval is the *peak time*. The intensity measured may then not be the *peak* effect, but rather some other intensity of effect reached anywhere during the rise to, or the fall from, the peak effect of the dose examined. One can well imagine the errors in potency values obtained from such spurious "ED_{50}s",—for instance, if the standard drug has a small and

the test drug a large peak time, and the potency ratio between the two drugs is formed by use of doses which are equieffective at the same rigid time interval from administration. According to the preceding discussion, potency evaluations by way of ED_{50} comparisons are, to say the least, highly problematic unless they include determination of peak time as the indispensable criterion of ED_{50} and define this value with the same biometrical elaborateness as the values presented as ED_{50}s. How Median Peak Time can be determined is indicated in the preceding example.

Summary. The intertwining of temporal and intensity features of drug effects and the necessity of its consideration in their quanti-tation are demonstrated by two examples: 1. "Duration of effect" requires reference to an endpoint of intensity of effect; it is then appropriately determined by "median duration" and suitable confidence limits. 2. The customary "median effective dose" is meaningless as a measure of potency unless determined at the time of peak effect; thus, determination of "median peak time" and its confidence limits is the prerequisite of ED_{50} determinations.

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Increased Excretion of Dehydroascorbic and Diketogulonic Acids by Rats After X-Ray Irradiation.*   (19954)

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Recent work has shown that rats excrete increased amounts of dehydroascorbic acid (DHA) and diketogulonic acid (DKA) in the urine when they are exposed to environmental temperatures of $0^\circ C(1)$. In order to establish whether this increased excretion of oxidized ascorbic acid (AsA) is a specific response to cold stress or a more general one obtained from stress of other kinds, adult albino rats were exposed to a second type of stress differing from the cold environment, namely, X-ray irradiation.

Experimental. Eight rats of the same inbred strain as those used in the cold experiments(1) with starting weights of about 200 g, paired as to sex, were used. The animals were placed 2 at a time in a flat wooden box and carried to the X-ray wing of the hospital, and then returned without treatment to the constant temperature animal room and placed in individual metabolism cages. The floors

and funnel portions of the cages were silicone-coated, and the 24-hour urine was collected and analyzed for AsA, DHA and DKA(2). Any possible effect of the trip in the irradiation box was thus reproduced in the controls. The control excretion was determined for 10-14 days, and then the same rats were taken back to the X-ray wing and exposed, 2 at a time, to a single, total body irradiation of 800 r, in air. (200 KV, 15 ma., 0.5 mm Cu and 3.0 mm aluminum filters, 50 cm target distance, 15 x 15 cm cone, and 25 r per minute dose rate). This dose represents an LD_{50} in 30 days in rats, and has been used by Smith and coworkers in metabolic studies(3). At each time of exposure, 2 new rats were carried along to start as controls. All irradiated animals survived the experimental period 10 days after exposure in apparent good health. The urinary excretion of AsA, DHA and DKA was determined exactly as in the control period.

Results of the analyses are summarized in Table I. As in the animals exposed to low

TABLE I. Twenty-Four Hour Urinary Excretion of Ascorbic, Dehydroascorbic, and Diketo-gulonic Acids by Rats before and after Exposure to 800 r X-Ray Irradiation.

Fraction determined	No. of analyses		Avg 24 hr excretion, μg with S.D.		
	Control	Irrad-iated	Control	Irradiated	"F"
AsA	89	77	347 ± 269	477 ± 490	4.64*
DHA	89	77	34 ± 37	88 ± 80	31.6 †
DKA	89	77	19 ± 38	63 ± 79	21.8 †

* Probability of variance ratio "F" less than .05, greater than .01.
† " " " " " " " .01.

environmental temperatures, the daily variation in excretion was great. The increase in AsA excretion was too slight to be statistically significant. The increases in the 2 oxidized forms were about 150% for DHA and 230% for DKA, roughly comparable to the increases obtained from cold stress, and statistically highly significant.

Discussion. The increased urinary excretion of oxidized forms of AsA in 2 forms of stress as widely different as cold environment and X-ray irradiation would seem to indicate that this may be a general response to stress. The mechanism of the production of DHA and DKA in the body, and the site of this production, remain to be established.

Summary. 1. Adult albino rats were exposed to 800 r single dose total body X-ray irradiation. 2. Urinary excretion of dehydroascorbic acid increased 150% after irradiation, diketogulonic acid excretion increased 230%. 3. The increase in excretion of oxidized ascorbic acid in the urine may be a general response to stress.

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Blood Volumes of Ducks Using Human Serum Albumin Labeled with Radioiodine.   (19955)

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For the past 70-odd years the literature has abounded with reports of various technics for blood volume determinations. One of the most widely used and undoubtedly one of the most precise methods involves evaluation of the plasma dilution of injected radioiodine (I^{131}) labeled human serum albumin(1,2). Using various technics blood volumes have not only been reported for the human, but also for the cat(3), dog(4), goat and horse(5), rabbit(6), and rat(7). The authors have in several of their investigations found a need for similar data on the duck, *e.g.*, in a more quantitative pathological evaluation of avian malaria in ducks(8), in a study of the normal hematology of the duck(9), in the evaluation of the hematological effects of toxic agents in the duck(10), and in a recently developed method for determination of the life of the duck erythrocyte(11).

Further study along several of these lines

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is in progress. Since apparently no studies of blood volume exist for the duck, we are reporting our findings.

Experimental. Radioiodine (I[131]) labeled human serum albumin (supplied by Abbott Laboratories, North Chicago, Ill.) was prepared in isotonic saline to concentration of about 1.5 μc/ml. Intravenous injections into the leg vein were made at a level of 0.3 to 0.6 ml/kg of body weight. 5 ml blood samples were withdrawn from the heart for the volume determinations, whereas 2 ml samples were taken from the leg veins of the larger ducks used in time-concentration studies. Withdrawals for the blood volume determinations were made within 15 minutes following injection. *Hematocrits* were determined using standard tubes with centrifugation at 2500 r.p.m. for 30 minutes. Radioassay was done on 1 ml samples of plasma after air drying in planchettes at room temperature. Standards were prepared by using labeled albumin diluted with human plasma. Radioassay technics used have been reported(12). Counting was sufficiently long to give a probable error of $\pm$ 1%. Calculations of the volumes were made as follows: Plasma volume (ml) = Total counts injected/Counts in 1 ml plasma, Blood volume (ml) = Plasma vol (ml)/100-hematocrit (%). White Pekin ducks, varying in age from 3 weeks to 1 year and in weight from 150 to 2038 g, were used. Food and water were available to them at all times. Gross *precipitation tests* were made by mixing varying portions of labeled albumin with duck plasma, incubating at 37°C, and

TABLE I. Mean Plasma and Blood Volumes with Stand. Errors of the Mean for 3 Groups of Ducks.

Wt, g	No. of ducks	ml plasma/ kg body wt	ml blood/ kg body wt
150- 450	24	69.4±1.52	107 ±2.34
700-1100	10	64.5±1.02	102.1±1.79
1100-2000	8	55.2±1.23	86.3±1.58

studying grossly and microscopically for evidence of precipitation. Estimate of the fraction of radioactive iodine in the injected material which was dialyzable was made by dialysis of the injected material against 3 volumes of isotonic saline and radioassay of the dialysate.

Results and discussion. The time-concentration studies on 5 large ducks are summarized in Fig. 1. It will be noted that the drop in concentration is rather sharp; however, it has been assumed that values taken within the first 15 minutes after injection will be suitable for volume determinations.

Data giving the mean plasma volumes, mean blood volumes and standard errors of the mean are listed in Table I. It will be noted that in the duck the blood volume per unit weight is larger for the smaller birds.

The results of the dialysis of labeled albumin revealed that 3.7% of the activity is dialyzable; since a large portion of even this small fraction could be expected to remain in the circulation for longer than 15 minutes, it has been assumed that the dialyzable fraction does not introduce a sizable error. All precipitation tests were negative.

Summary. Blood volume determinations were performed on 42 ducks of various weights, using radioiodinated human serum albumin. The blood in ml per kg body weight in young ducks weighing 150-450 g is 107 ± 2.34. For the intermediate group weighing 700-1100 g it is 102 ± 1.79. In young adult birds weighing 1100-2000 g it is 86.3 ± 1.58. Several supplementary procedures were carried out to test the propriety of this method in the duck.
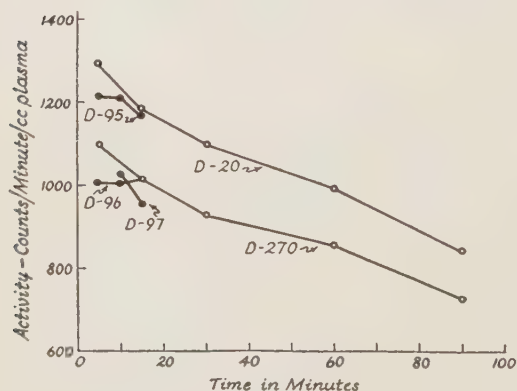
FIG. 1. Time-concentration studies of radioiodinated human serum albumin in 5 adult ducks.

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Concentration Changes in Urinary Electrolytes Produced by Mercurial Diuretics.* (19956)

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Mercurial diuretics usually produce an increase in sodium and chloride excretion by increasing the urine volume and by increasing the urinary electrolyte concentrations. Under certain conditions mercurials can decrease urinary sodium and chloride concentrations. An attempt will be made to define the conditions which determine these different changes in urinary electrolyte concentrations.

Methods. Both anesthetized and unanesthetized dogs were used. Anesthesia was produced by means of an intravenous injection of 30 mg of pentobarbital per kg and the depth of anesthesia was kept constant by infusing 0.3 to 0.4 mg of pentobarbital per kg per minute. The dogs received infusions of 3% glucose or 2% sodium chloride. The constant rate of infusion was varied between 1.8 and 17 cc per minute per m^2 of body surface.

Para-aminohippurate (PAH) clearance at low plasma concentrations was considered to be the renal plasma flow (RPF) while inulin or creatinine clearance was used as an index of glomerular filtration. Sodium and potassium were determined in plasma and urine by means of an internal standard or a Beckman flame photometer. Chloride was determined by the method of Van Slyke and Hiller(1). In general, the methods used were essentially similar to those used in a previous study(2). The experiments on unanesthetized animals were conducted on trained female mongrel dogs weighing 18 to 22 kg. Isotonic saline infusions were given into a leg vein and blood specimens were obtained from an external jugular vein. Urine was collected by means of a self retaining soft rubber catheter. About one hour following the start of the saline infusion, 0.1 unit of pituitrin was injected intramuscularly and enough pituitrin was added to the infusion fluid to give a constant injection rate of about 200 milliunits per hour. After pituitrin had been injected for approximately one hour, 8 mg per kg of mersalyl was given together with glutathione or cysteine. The procedures and analytical methods used in these experiments were essentially the same as those described for the anesthetized group

of animals. The mercurial diuretics used were mersalyl[||] or Esidron acid.[¶] Both substances were used without aminophylline and the acids were dissolved in isotonic saline by means of sodium hydroxide. In most instances the mercurials were given together with cysteine or glutathione in a 1:1 molar ratio(3,4). Three experiments were conducted on rats using essentially the same technic as described by Burn(5) for the assay of antidiuretic hormone. Water was given by a stomach tube (5 cc per 100 g of body weight) followed by the subcutaneous injection of 2 milliunits of pitressin per 100 g of body weight. Mersalyl and cysteine (1:1 molar ratio) were given intramuscularly in a dose of 1 or 3 mg per 100 g of body weight

FIG. 2. Mersalyl diuresis during a pituitrin infusion. ♀ dog 14.8 kg, unanesthetized; infusion of .86% sodium chloride at 7 cc/min./m² body surface. At Pit.: start i.v. infusion of pituitrin 10 milliunits/kg/hr. At Hg: intrav. inj. of 30 mg of mersalyl and cysteine (1:1 molar ratio)/kg. BAL: 8 mg of 2,3 dimercaptopropanol/kg intrav.

FIG. 1. Effect of mersalyl on urine flow and urinary sodium and chloride concentration during an infusion of .25% sodium chloride in 3% glucose. ♀ dog 14.3 kg, pentobarbital anesthesia, intrav. infusion of .25% sodium chloride in 3% glucose at a rate of 6.4 cc/min./m² body surface. At Hg inj. of 25 mg of mersalyl and cysteine (1:1 molar ratio) per kg. At BAL 10 mg of 2,3 dimercaptopropanol/kg intramusc.

[||] Kindly supplied by Sterling Winthrop Research Institute, Rensselaer, N. Y.

[¶] Kindly supplied by Ciba Pharmaceutical Products, Summit, N. J.

15 to 20 minutes before the administration of the water and pitressin. The time required for the excretion of 50% of the water load was calculated and was used an an index of activity.

Results. The results were essentially the same in the anesthetized and unanesthetized animals. Mersalyl and Esidron acid were shown to have similar qualitative and quantitative actions on urinary flow and electrolyte excretion(2) and in the present study most of the experiments were conducted with mersalyl.

Mercurial effects during the infusion of hypotonic saline. The infusion of 3% glucose or hypotonic saline (0.2% NaCl in 3% glucose) resulted in low urinary electrolyte concentrations, minimal values being attained within 2 to 3 hours after the start of the infusion. The mercurial produced an increase in urine flow and an increase in the urinary sodium and chloride concentration. This increase in sodium and chloride concentration approached and in some instances even ex-

TABLE I. Effect of an Injection of Mersalyl into the Left Renal Artery on Urine Flow and Electrolyte Excretion. Male dog 23 kg, pentobarbital anesthesia, I.V., infusion of 3.8 cc/min. of a 3% glucose solution. 30 mg of mersalyl was injected into the left renal artery. Determinations were made on separate kidneys.

Time, min.	Urine flow, cc/min.		Glomerular filtration, cc/min.		Plasma sodium, mM/l	Urinary Na, conc., mM/l		Na excretion, microequiv./min.	
	Right	Left	Right	Left		Right	Left	Right	Left
0– 30	.17	.16	22.8	25.3	126	9	7	1.53	1.12
30– 50	.68	.80	21.5	25.8	128	1.5	1.5	1.02	1.20
50– 52			Mersalyl 30 mg into left renal artery						
50– 70	.70	.80	28.2	31.1	127	.90	.94	.63	.75
70– 85	.17	2.43	27.5	23.7	126.5	17.8	152	3.03	369.4
85–100	.27	2	29.2	28.2	127	3.1	144	.83	288
100–115	.25	1.13	30.7	26	128	2.6	124	.65	140
115–130	.17	.48	25.7	30.5	125	4.1	123	.69	59.4
130–145	.20	.23	26.3	31	122	6.1	93	1.22	21.4
145–160	.25	.30	29.5	28.2	124	4.2	85	1.05	25.5

TABLE II. Action of Mersalyl on Urinary Sodium and Chloride Concentration and Excretion during a Hypertonic Saline Infusion. Male dog 12.3 kg, pentobarbital anesthesia. Infusion of 2% sodium chloride intravenously, at a rate of 12.5 cc/m² body surface/min. All values are based on one m² of body area.

Time, min.	Urine flow, cc/min.	GFR, cc/min.	Plasma Na, mM/l	Urinary Na, conc., mM/l	Na excreted, mM/min.	Plasma Cl, mM/l	Urinary Cl, conc., mM/l	Cl excreted, mM/min.
0– 20	14	123	161.3	198.5	2.78	125	185	2.59
20– 40	13.4	125	165.8	232	3.10	129	222	2.97
	Mersalyl with cysteine, 25 mg/kg i.v.							
40– 60	11.28	98.3	165	233.7	2.64	129	229	2.58
60– 80	10.93	97	165.3	224.2	2.45	129	221	2.42
80– 90	17.5	102.6	169.4	206	3.61	134	204	3.57
90–100	22.2	100.9	171.9	204	4.53	136	201	4.46
100–110	22.8	98.3	174.9	215	4.90	139	213	4.86
110–120	19	87.8	173.7	210	4	139	210	3.99
	BAL, 5 mg/kg i.v.							
120–140	10	73.4	183.6	215	2.14	150	214	2.14
140–160	6	91.6	181.8	235.6	1.40	149	232	1.39
160–180	9.8	99.5	182	236.8	2.32	149	232	2.27

ceeded that of plasma (Fig. 1). This overshoot was more commonly seen with chloride than with sodium probably because plasma chloride was lower than the plasma sodium concentration. In 4 of our experiments urinary sodium concentration exceeded that of plasma by 20 to 40 milliequivalents following the mercurial injection. In 5 successful experiments the mercurial was injected into the left renal artery and produced a unilateral diuresis(6). In 2 of these experiments the injection of 20 mg of mersalyl into the left renal artery resulted in a unilateral diuresis and increase in sodium concentration which

exceeded that of the plasma by about 25-30 milliequivalents (Table I).

Mercurial effects during the infusion of hypertonic saline. Two per cent sodium chloride was infused into 5 anesthetized dogs. In 3 of these the urinary sodium and chloride concentration was above that of plasma by about 50 to 70 milliequivalents. The injection of the mercurial resulted in a reduction of the urinary sodium concentration; however, the mercurial induced volume changes resulted in a net increase in sodium and chloride excretion. In all 3 instances the urinary sodium and chloride concentration after the mercurial

TABLE III. Action of Mersalyl on Pitressin Antidiuresis in the Rat. All animals received 50 cc/kg of distilled water by mouth. Pitressin was given subcutaneously while mersalyl with cysteine (1:1 molar ratio) was given intramuscularly. Each group consisted of 3 rats weighing 200 to 265 g, 3 experiments were conducted.

	50% excretion time in min.	Avg
Control	75, 68, 70	71
Mersalyl, 10 mg/kg	60, 63, 68	64
" 30 "	67, 70, 59	66
Pitressin, 20 m units/kg	143, 132, 137	137
Mersalyl, 10 mg/kg + pitressin, 20 m units/kg	135, 129, 135	133
Mersalyl, 30 mg + pitressin, 20 m units/kg	125, 135, 130	130

remained well above that of plasma (Table II).

Mercurial effects during pituitrin infusion. Four anesthetized and 3 unanesthetized dogs were infused with isotonic saline at a rate of about 7 to 15 cc per minute per m² of body surface. After an adequate control period pituitrin was added to the infusion field. In both the anesthetized and unanesthetized dogs, the pituitrin effects were variable. Pituitrin antidiuresis was observed in 3 dogs of this series (Fig. 2) and in the remaining dogs, pituitrin increased urine flow. However, pituitrin, regardless of the urine volume response, always increased the concentration of sodium and chloride ions in the urine and in 3 instances it raised the concentration of these ions appreciably above those of plasma sodium and chloride concentrations. The administration of a mercurial after pituitrin increased urine flow and the net sodium and chloride excretion in every one of our experiments. In those experiments in which urinary sodium and chloride concentrations were above those of plasma, the mercurial produced a significant reduction in the urinary concentration of these ions. However, following the mercurial the urine was still hypertonic to plasma as regards sodium and chloride (Fig. 2).

Pituitrin antidiuresis and mersalyl in rats. In these experiments 10 mg and 30 mg of salyrgan with cysteine per kg were given intramuscularly followed by the oral test dose

of water and the subcutaneous injection of pitressin. It can be seen from Table III that the mercurial in either dose decreased slightly the 50% excretion time of the water load. Pitressin alone produced a marked increase in the excretion time while pitressin and the mercurial given together had about the same effect as pitressin alone. The effects of the mercurials' on urinary potassium excretion were variable. As has been described by Mudge *et al.*(7), mercurials increased net potassium excretion when urinary potassium was low and decreased it when it was high. However, under all conditions of the present experiments urinary potassium concentration was decreased by the mercurial.

Discussion. The results clearly indicate that the mercurial does not block the ability of the kidney to produce a hypertonic urine as regards sodium and chloride nor does it block the antidiuretic effect of pitressin. These findings are in agreement with those of Weston *et al.*(8) and Pitts *et al.*(9). It must be concluded that the mercurials do not interfere with those renal tubular mechanisms involved in the production of a hypertonic urine nor do they impair the ability of the tubules to respond to pitressin. In contrast the mercurial diuretics counteract the effects of the salt retaining adrenal cortical hormones(10). A possible mechanism of the mercurial diuretics suggested by Mudge, Foulks, and Gilman(11), and Weston *et al.* (8) could be the inhibition of proximal tubular reabsorption of water, sodium and chloride. In this part of the tubule, the reabsorption of fluid and salt has been postulated to be isosmotic to glomerular filtrate. The inhibition of this reabsorptive mechanism would thus result in the addition to the control urine of electrolytes equivalent in amounts to those present in glomerular filtrate. Thus if the urine is hypotonic to plasma as regards sodium and chloride, the addition of glomerular filtrate should increase the above urinary electrolyte concentrations. On the other hand, if the control urinary sodium and chloride concentration is hypertonic to plasma the addition of glomerular filtrate should decrease the urinary sodium and chloride concentrations. If this hypothesis is correct, urinary

sodium and chloride concentrations after the mercurial should not exceed those of plasma. However, we have demonstrated that the urinary sodium concentration can increase to levels significantly higher than those of plasma (Fig. 1). A possible cause for this overshoot phenomenon could be the release of antidiuretic hormone by the mercurial and the additive effects of these 2 substances could explain this overshoot phenomenon. Pack (12) and Kuschinsky(13) have shown that following the injection of mercury into rats the concentration of oxytocic and antidiuretic principles of the pituitary gland are reduced. If the release of antidiuretic hormone were the cause of the overshoot shown in Fig. 1 this overshoot should not occur if a unilateral diuresis is produced by injecting the mercurial into one renal artery. This overshoot can occur under the above experimental conditions and it must be concluded that this phenomenon is due to some direct action of the mercurial on the kidney. Another possible explanation could be that the mercurial sensitized the kidney to circulating antidiuretic principle. The experiments on rats reported here do not support this explanation (Table III). The above findings make it rather difficult to explain the effects of mercurials as being on either the proximal or distal convoluted tubule. The possibility of more than one site of renal action of mercurials must be considered.

Summary. 1. The mercurial diuretics increase sodium and chloride concentration in the urine when these ions are hypotonic to plasma. The mercurial induced increase in the urinary sodium and chloride concentrations can exceed those of plasma by a significant amount. 2. When urinary sodium and chloride concentrations are above those of plasma the mercurials reduce the concentration of these ions significantly. The ability of the kidney to produce hypertonic urine is not inhibited by mercurial diuretics. 3. Pituitrin induced antidiuresis and hypertonicity of the urine are not abolished by maximally effective doses of mercurial diuretics.

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Mating Types in *Stylonychia putrina*. (19957)

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Although conjugation in ciliated Protozoa was long extensively studied, especially by Maupas(1), mating types were first discovered by Sonneborn(2). It is now known that mating types exist in *Paramecium aurelia*(2,3), *P. bursaria*(4-6), *P. caudatum*(7-11), *P. multimicronucleatum*(11), *P. trichium*(12), *P. calkinsi*(12-14), and *Euplotes patella*(15). In several of these species, a number of varieties have been recognized, each with its distinctive mating types; and the number of interbreeding mating types within a variety

is 2 in some cases, 4, 6, or 8 in others. The present paper reports 5 mating types in *Stylonychia putrina* Stokes, like Euplotes a hypotrichous Ciliate.

Material and methods. The clones of *Stylonychia putrina* used in the experiments reported here were collected at Lake Arrowhead in Southern California on August 30, 1949; September 25, 1949; June 27, 1950; and September 24, 1950. In all, 41 clones were isolated and established. The culture medium and methods were essentially those described by Sonneborn and Dippell(16).

Mating conditions and behavior. In testing for mating type, the clones were mixed during the first half of the forenoon. Pairing occurred only if the animals were numerous in the mixtures. The tendency to form pairs appeared to be stronger immediately succeeding food exhaustion. For this reason it was found advisable to add a drop of culture medium to each mixture, as made, as Kimball(15) did with *Euplotes patella*. If the animals were numerous, this amount of food was soon used up and pairing followed. When different mating types were mixed there was no immediate agglutinative reaction such as is found in Paramecium, but sometime later the animals gathered closely together in the bottom of the depression slide. Their normal jerking movements were accelerated; they appeared to be greatly excited. When pairing began, two animals faced each other and touched peristomes. After a time they became attached and twisted around till they lay side by side. Grell(17) observed similar prepairing behavior in *Stylonychia mytilus*.

In these experiments, pair formation usually began soon after noon and ceased around 5 or 6 o'clock in the evening. Occasionally pairing would begin later and would continue into the night. This statement is not intended to imply diurnal periodicity. This question requires further study. Pairs usually remained attached for about 24 hours but there was considerable variation in this.

Conjugation was observed in certain mixtures of 26 of the 41 clones tested. In preliminary work 5 of these clones were found to conjugate with each other in all possible combinations. Since selfing was never observed in any of the 5 clones either in the culture vials which were under constant observation, or in unmixed controls set up under the same conditions as the experimental mixtures, it is obvious that 5 mating types were present. On being tested the other 21 clones were found to belong to the same 5 mating types, which have been designated as mating types A, B, C, D, and E of Variety I of *Stylonychia putrina*. In one of the clones selfing was observed, a few pairs being frequently seen in the culture vial. However, when this clone was mixed with clones of the 5 mating types, there would be many pairs in mixtures with mating types A, C, D, and E, but in the mixture with a clone of type B, the number of pairs would not be significantly greater than in the unmixed control. This clone was therefore assigned to mating type B. Fifteen of the 41 clones tested failed to conjugate with any of the 5 clones given above and various attempts to get them to conjugate with each other also failed. This complete lack of reactivity on the part of over one third of the clones tested remains to be explained. They may belong to another variety, they may be sexually immature, or their lack of reactivity may be due to some other unknown condition.

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Pharmacologic Action of β-Phenylethylglucosamine.*   (19958)

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The quantitative difference in activity between the optical isomers of various sympathomimetic amines has been widely investigated. The present report concerns the changes in pharmacologic activity of optically inactive β-phenylethylamine which result when it is conjugated with an optically active sugar moiety.

Materials and methods. β-Phenylethylglucosamine (PEGA) was prepared by the method of Pigman *et al.*(1). It was a white crystalline solid (M.P. 82°) which gradually darkened on standing. *Analysis.* Calculated for $C_{14}H_{19}O_5N$; N, 4.98. Found: N, 5.06. Mutarotation studies of PEGA in water were performed with standard polarimetric technics. Freshly prepared aqueous solutions of PEGA had a specific rotation $\alpha]_d^{20} -21°$ however; upon standing at room temperature for 15 minutes, the value fell to $\alpha]_d^{20} -15°$ and remained constant over a period of 2 hours. Heating the solution for 1 hour at 50° yielded a value of $\alpha]_d^{20} +8°$ which remained constant over a period of 1 hour. Heating PEGA solutions at temperatures of above 60°C, however, very quickly hydrolyzed the glucosamine. Toxicities were calculated by the method of probits following intraperitoneal injections of aqueous solutions in groups of 20-30 white mice. The effects on carotid arterial blood pressure were determined in pentobarbitalized dogs following intravenous injections of aqueous solutions of the drugs.

Results. The toxicities of the two forms of PEGA (+8 and −15) and of β-phenylethyl-

TABLE I. Acute Toxicity Study.

Compound	LD_{50} (mg/kg)	LD_{50} moles $\times 10^3$/kg
PE	366	1.47
PEGA (+8)	426	1.53
PEGA (−15)	535	1.89

amine hydrochloride (PE) are compared in Table I on both a milligram and molar basis. Whereas PEGA (+8) and PE were apparently not different, PEGA (−15) was definitely less toxic. The observable responses in the mice were essentially the same for all materials although PE appeared to exert its effects more rapidly than either form of PEGA. Following the injection of lethal doses of all materials the mice initially were quite aggressive[†] then followed a period of relative inactivity which merged into fine tremors, later coarse tremors and finally asymmetrical convulsions. Upon cessation of respiration, opening the chest cavity revealed the heart was still beating.

In their effects on the dog's blood pressure, PEGA (+8) and PE produced qualitatively and quantitatively similar pressor responses and were roughly 1/300 as potent on a weight basis as epinephrine. PEGA (−15), however, appeared to be only about 1/3-1/5 as active. Qualitatively, PEGA (−15) in low dosage ranges (up to 2 mg/kg) produced a more prolonged rise in pressure than did PEGA (+8) or PE at a dose of equal pressor height. Doses of PEGA (−15) in high dosage ranges (5 mg/kg and larger) gave responses qualitative-

* Preliminary report in *J. Pharm. and Exp. Therap.,* 1951, v103, 350.

† The term 'aggressive' here denotes the fact that mice were observed to run about the cage biting one another.

ly like those of PEGA (+8) and PE. The blood pressure responses to epinephrine were unchanged by the prior administration of any of these materials.

Discussion. The combination of glucose with β-phenylethylamine appears to change the qualitative toxicity of PE relatively little. However, quantitatively, the combination does appear to delay the onset of toxicity effects, and in the case of the levorotatory compound, PEGA (−15), molar toxicity is actually decreased. The blood pressure studies tend to confirm that PEGA (−15) is less active and more prolonged in its action than PE or PEGA (+8).

The quantitative change in activity that occurs in PEGA (−15) may be contrasted with the results of Swanson *et al.*(2). These workers in a study of the effect of optically active isomers of acids in combination with sympathomimetic amines, concluded that the configuration of the acid did influence the activity of optically active amine salts but did not influence the activity of optically inactive PE. While the degree of dissociation

(ionization) of the amine salt as contrasted with the relatively undissociable covalent linkage of the glucosamine is great the glucosyl group does not act in the same manner as simple nitrogen substituents. Bovet(3) reports that all N-substituted PE derivatives in which the substituent was ethyl or larger were sympatholytic; none of the present materials is sympatholytic.

Summary. The glucosyl derivatives of β-phenylethylamine have been synthesized and compared with β-phenylethylamine with respect to toxicity and pressor effects. The levorotatory β-phenylethyl glucosamine is less toxic and more prolonged in its action than β-phenylethylamine or the dextrorotatory glucosamine.

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Distribution of P^{32} in Tissues of Normal Animals.*   (19959)

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Many investigators are using radioactive phosphorus in biological studies but there is very little recent data available on the distribution of P^{32} in the various tissues. The most extensive investigations available up to the present time deal with the use of radioactive phosphorus in connection with the phospholipid metabolism, which was summarized by Chaikoff(1). As a preliminary study in connection with the effect of selenium poisoning on phosphorus metabolism we have

investigated the distribution of P^{31} and P^{32} in the tissues of normal animals. In this review we would like to report our findings. Detailed studies on liver, kidney, spleen, heart, and brain will be given in subsequent reports.

Methods. Normal adult Sprague-Dawley male and female rats were used as experimental animals and injected intraperitoneally with 2.6684×10^8 counts per minute of P^{32}. The P^{32} (H_3PO_4) was received from Oak Ridge in weak HCl solution which contained 0.024 mg P per millicurie and was neutralized with NaOH before injection. Animals were sacrificed under nembutal anesthesia at 1, 2, and 12 hours after the administration of the isotope. The results are the average of 5 or

TABLE I.
Distribution of P³¹ and P³² in the Tissues of Normal Animals at Various Time Intervals.

Organ	1 hr			2 hr			12 hr		
	P³¹, mg %	P³² *, g %	S.A.† × 10⁴	P³¹, mg %	P³², g %	S.A. × 10⁴	P³¹, mg %	P³², g %	S.A. × 10⁴
Brain	309.6	.2	3.43	320.1	.3	5.25	299.3	.09	8.33
Heart	206.4	.33	87.44	199	.39	104.55	205.3	.19	42.15
Lung	259.3	.39	41.81	260.3	.70	82.24	264.8	.37	39.41
Liver	325.5	1.50	148.9	319	1.86	171.14	325	.99	84.72
Spleen	294.4	.48	50.38	299.2	.52	67.55	294	.50	52.60
Kidney	282.4	.94	114.28	287	1.27	130.5	290.8	.58	51.42
Stomach	197	.37	48.01	189.3	.52	78.13	185.2	.32	47.46
Small intestine	212.5	.55	65.86	201.3	.83	118.26	201.5	.42	53.79
Large intestine	178.8	.38	60.14	185	.71	108.99	181.2	.28	41
Uterus	184	.24	54.78	187.8	.70	108.66	193.4	.64	94.43
Testicle	193.9	.05	22.6				180.6	.09	42.1
Muscle	199.3	.14	14.32	201.4	.22	37.32	197.2	.17	22.52
Serum	7.5	.9	280.85	8.7	1.6	409.32	9.8	.03	88.31

* % of the administered dose recovered/g tissue.
† Specific activity = c/m/mg/P. The figures listed are to be multiplied by 10⁴ to obtain the specific activity.

more rats in each time interval studied. The weighed tissues were divided in two parts and duplicate tissues were ashed with 10N H_2SO_4 and HNO_3 and cleared with a few drops of 30% hydrogen peroxide. The ashed tissues were diluted to volume and aliquots were used for the determination of the total phosphorus and the radioactive phosphorus. The blood was obtained by cardiac puncture and serum and cells were separated by centrifugation. The protein from the serum was precipitated with 10 percent trichloroacetic acid and the results presented are the acid-soluble phosphate of the serum. The total phosphorus was determined according to the method of Fiske and Subbarow(2). The activity of P³² was measured on dried aliquot of the sample in a Geiger-Muller counter. All samples were corrected for decay and the values reported are in terms of the activity at the time of injection. The specific activity is defined as the c/m/mg P.

Results. It appears from the data presented in Table I that the radiophosphorus uptake and the specific activity of the various organs show considerable variation. It is apparent that a considerable amount of the inorganic phosphate entered into the tissues at the end of the first hour. The optimum uptake in the tissues was reached at the end of the second hour. The rapid turnover rate of phosphorus in the serum is indicated by the study of the specific activity of the serum at

1, 2, and 12 hours. The specific activity at the end of the first hour was 280.85 x 10⁴ and increased to 409.32 x 10⁴ at the end of the second hour but at the end of 12 hours it decreased to 88.31 x 10⁴. It is quite evident from the data that the phosphorus from the serum is rapidly transferred to the various tissues of the body.

According to some investigators the difference in the uptake and turnover rate of phosphorus in the tissues is related to the permeability of the tissue cells to phosphorus. Lungsgaard(3) studied the rate of rejuvenation of phosphorus compounds in the liver using radioactive phosphorus and from his studies concluded that there is a rapid rate of rejuvenation of the acid-labile phosphoric esters and penetration of the phosphate ion into the liver cell. We have obtained similar results in our studies on the phosphorylated ester in liver which will be reported in a future publication. Our present data are based on the radioactive phosphorus uptake and specific activity of the total liver, which contains many phosphorylated compounds which are rejuvenated at various rates. However, on the total tissue basis the liver utilized the largest amount of radioactive phosphorus among all the organs studied.

The different anatomical parts of the gastrointestinal tract showed considerable variation as to the uptake and specific activity of radiophosphorus. The small intestine with its

highly selective absorption contained the highest percentage of P^{32} and the highest specific activity followed by the large intestine and stomach. It appears that the uptake of phosphates by the gastrointestinal tract is directly related to the physiological function of the segment of this organ.

Although the brain and liver have approximately the same amount of organic phosphates, the liver contained almost eight times as much radioactive phosphorus per unit weight and time as the brain. The extent which phosphorus is taken up by the cells appears to depend on not only the total amount of phosphorus present in the tissues but on the permeability of cells to the phosphate ion. The brain seems to be almost a closed system with regard to the interchange of phosphorus with the body fluids.

The specific activity and the gram % uptake in the heart is much higher than in the skeletal muscle. However, the heart was not perfused and the difference may be due to the presence of residual blood within the organ. The permeability of muscle cells to inorganic phosphate is low according to the earlier investigators. Hevesy and Rebbe(4) showed that the phosphate penetrates the skeletal muscle cells very slowly and concluded from their studies that the specific activity of the total tissue inorganic phosphate, which includes both the extracellular and intracellular components, is not the true indication of the specific activity of the inorganic phosphate within the cell. Kalckar et al.(5) by using perfusion technic studied the rate of penetration and the rate of rejuvenation of organic phospho compounds of skeletal muscle and liver. They have estimated that the rate of penetration of phosphate in muscle was approximately one gamma of P per minute per g of muscle. However, as in our data, when the results are based on the total tissue uptake and the specific activity without regard to the intracellular and extracellular phosphorus the rate of turnover of phosphorus in the skeletal muscle is very low.

The radioactive phosphate administered in inorganic form is readily incorporated into the kidney. The rate of appearance and disappearance based on the percent uptake and specific activity of P^{32} in this tissue appears to be similar to that of liver but the magnitude is at a lower level. Perlman et al.(6) measured the deposition of phospholipid in the kidney after a single injection of P^{32} and found that the deposition and disappearance of the labeled phospholipid was slower in the kidney than in the liver and intestine.

The P^{32} distribution and specific activity in the spleen showed very little change at the time intervals studied.

The specific activity and gram % uptake of P^{32} in the lung showed an increase at the end of the second hour and at the end of 12 hours there was considerable decrease indicating a high turnover rate in this organ.

The male and female sex organs showed considerable variation as to uptake and specific activity. The uterus showed a higher percent uptake than the testicle. This would indicate that the rate of phosphorus metabolism in the testicle is very low.

In comparing the distribution of alkaline phosphatase in the various tissues as was reported by Kabat and Furth(7) with the differences in the uptake of the radioactive phosphorus in the tissues studied we have found it of special interest that tissues which contain large amounts of alkaline phosphatase have a higher specific activity and higher percent of P^{32} uptake than tissues which are low in alkaline phosphatase. Whether there is any correlation between these two factors it is difficult to state since the function of alkaline phosphatase in many tissues is not clearly understood.

Summary. The distribution and the specific activity of P^{32} in the various organs of normal animals was studied at 1, 2, and 12 hours after intraperitoneal administration of the isotope. The P^{32} was rapidly taken up by the serum and distributed in the various organs. It appears that the distribution of P^{32} was not directly related to the amount of phosphorus present in the tissue but depended on the permeability of the cells to the phosphate ion.

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Comparison of Eosinophil and Circulating 17-Hydroxycorticosteroid Responses to Epinephrine and ACTH.*   (19960)

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Since relatively pure adrenocorticotropin (ACTH) has become available, the eosinophil response test has emerged as a commonly used and simple method of assessing adrenocortical function. The usual response to the injection of ACTH, Compound E, Compound F, epinephrine or insulin, and to non-specific stress stimuli is characterized by a reduction in the number of circulating eosinophils(1). This eosinopenic response to the administration of ACTH has been used as a major criterion indicating an intact adrenal cortex, with the absence of eosinopenia interpreted as indicating adrenal cortical insufficiency. It has been assumed that in order to elicit an eosinopenic response to epinephrine or non-specific stress stimuli an intact anterior pituitary and adrenal cortex must be present. The postulation has been advanced that these stimuli act through the hypothalamus upon the anterior pituitary causing release of ACTH, which in turn stimulates the adrenal cortex to secrete its hormones(2). The hormones secreted by the adrenal cortex which are generally believed to effect the eosinopenia are the 17-hydroxycorticosteroids(3-7). However, because epinephrine eosinopenia occasionally has been induced in states of impaired function or absence of the adrenal cortex(1,8) the validity of this assumption has been questioned.

Recent development of methods allowing direct measurement of circulating adrenal hormones provides a technic permitting a more definitive evaluation of the relationship between eosinophil and adrenal steroid responses to a stimulating agent. In the present study the circulating plasma concentration of 17-hydroxycorticosteroids has been measured and the effects of ACTH or epinephrine injection on the levels of these steroids and of eosinophils have been compared in children. It should be emphasized that the influence of specific disorders on the type of adrenal response is of secondary interest only, and that the point of primary concern is the comparison of the responses of circulating eosinophils and 17-hydroxycorticosteroids.

Methods. Healthy children and children with various disorders have been used in this study. Responses of eosinophils and 17-hydroxycorticosteroids to the injection of .01 mg/K of epinephrine hydrochloride or 25 I.U. ACTH were studied. Eosinophils were determined by the method of Randolph(9) and circulating plasma 17-hydroxycorticosteroids by Nelson and Samuels' modification(10) of their method for whole blood(11). Total eosinophil counts were done at 0, 1, 2 and 4 hours. The minimum value obtained, expressed as per cent decrease from the control value, is used in the data of the tables. The circulating plasma 17-hydroxycorticosteroid values were obtained at 0 and 2 hours, because

TABLE I. Mean Response of 17-Hydroxycorticosteroids and Eosinophils to Epinephrine.

Group	No. pts.	Eosinophils		17-hydroxycorticosteroids (µg %)				
		No. response tests	% change, mean ± SE_M	No. pts.	No. response tests	Mean 0 hr value	Mean 2 hr value	Change, mean ± SE_M
Congenital adrenal hyperplasia	5	17	−59 ± 3.5	5	8*	6.6	7.8	1.2 ± 1.56
Rheumatic diseases	13	19	−21 ± 9.9	13	18	15.5	15.4	−.1 ± 2.73
All cases except congenital adrenal hyperplasia	41	48	−52 ± 4.7	30	35	11.7	12.6	.9 ± 1.37

* Some of these patients were receiving maintenance cortisone therapy at the time of these measurements. Since the technic employed does not differentiate between endogenous and exogenous 17-hydroxycorticosteroids the values, which are higher than expected, probably reflect measurements of the exogenous hormone(13).

TABLE II. Mean Response of 17-Hydroxycorticosteroids and Eosinophils to ACTH.

Group	No. pts.	Eosinophils		17-hydroxycorticosteroids (µg %)				
		No. response tests	% change, mean ± SE_M	No. pts.	No. response tests	0 hr	2 hr	Mean change, µg %
Congenital adrenal hyperplasia	5	35	−26 ± 4.9	5	12*	6.3	6.3	0 ± 1.23
Rheumatic disease	11	18	−36 ± 8.4	13	19	15.8	41.8	26 ± 4.71
All cases except congenital adrenal hyperplasia	58	72	−49 ± 3.7	19	30	13	36.5	23.5 ± 3.64

* These levels also include those obtained during periods of cortisone administration and thus reflect the influence of the exogenous hormone. (See footnote, Table I.)

concentrations of these steroids reach peak values within 2 hours after injection of ACTH(12).

Results. Table I shows the responses of eosinophils and 17-hydroxycorticosteroids to epinephrine in different groups of children. In none of the groups shown is there any significant mean elevation in the concentration of the 17-hydroxycorticosteroids in response to epinephrine. In contrast to this there is a satisfactory eosinophil response in all groups except that group with rheumatic disease. In this latter group the mean reduction of eosinophils is only 21 ± 9.9%.

The group of patients with congenital adrenal hyperplasia is separated from the other groups in this table because these patients apparently are unable to produce elevations of their 17-hydroxycorticosteroid concentrations even in response to ACTH injection (13). However, even in the group excluding these patients with congenital adrenal hyperplasia there is no response of 17-hydroxycorticosteroids to epinephrine.

The general responses of eosinophils and of 17-hydroxycorticosteroids elicited by ACTH in different groups of patients are illustrated and compared in Table II. In patients with congenital adrenal hyperplasia there is a mean 26% reduction of eosinophils but no elevation of 17-hydroxycorticosteroids. In the group of patients with rheumatic disease there is a most satisfactory elevation of 17-hydroxycorticosteroids in response to ACTH but an average eosinophil reduction of only 36%. The average of all ACTH response tests done, with the exception of the adrenal hyperplasia group, shows an eosinophil reduction of 49% and an adequate elevation of the 17-hydroxycorticosteroid level.

In Table III selected cases are presented to show that an eosinopenic response to ACTH may occur in the absence of 17-hydroxycorticosteroid elevations. In all of these children there is an adequate eosinopenic response without a corresponding elevation of 17-hydroxycorticosteroid concentrations.

Table IV shows data of 4 children whose

TABLE III. Response to ACTH. Illustrative cases (8 patients) showing adequate eosinopenia but poor 17-hydroxycorticosteroid response.

Eosinophils (% change)	17-hydroxycorticosteroids (μg %)	
	0 hr	2 hr
−64	11	5.4
−58	7.7	6.5
−45	13.9	11.1
−42	11.9	7.8
−69	2.5	3.1
−66	17.4	13.3
−66	13.6	14.4
−60	7.6	10
Mean −59%	10.7	9

TABLE IV. Response to ACTH. Illustrative cases (4 patients) showing poor eosinophil response with adequate 17-hydroxycorticosteroid elevation.

Eosinophils (% change)	17-hydroxycorticosteroids (μg %)	
	0 hr	2 hr
+33	12.6	55
+37	10.7	29.9
−12	5.2	63.5
−14	13.5	50.5
Mean +11%	10.5	49.7

responses to ACTH are opposite to those shown in Table III. Here are seen, on simultaneous determinations, poor eosinophil responses but adequate 17-hydroxycorticosteroid responses to ACTH. Thus, even following the administration of ACTH, under certain circumstances an eosinopenia need not be accompanied by a rise in 17-hydroxycorticosteroids; conversely, 17-hydroxycorticosteroid elevations need not be accompanied by an eosinopenia.

Discussion. The following evidence has been used to support the general belief that ACTH and epinephrine induced eosinopenia is mediated by the secretion of 17-hydroxycorticosteroids by the adrenal cortex: 1) Administration of ACTH, 17-hydroxycorticosterone or cortisone to the intact animal or man causes an eosinopenic response, whereas administration of desoxycorticosterone acetate does not(4), 2) *in vitro* incubation of blood with cortisone results in an eosinopenia(5), and 3) in man the adrenal cortical hormone secreted consists primarily of 17-hydroxycorticosterone(6,7). This evidence is highly suggestive, but confirmation by the use of direct technics is lacking.

In general, following the injection of ACTH in human subjects there is an adequate response of both eosinophils and circulating 17-hydroxycorticosteroid plasma concentrations, a reduction of the former and an elevation of the latter. However, certain exceptions to this generalization have been observed; some patients have exhibited a satisfactory eosinopenia without a concomitant increase in circulating 17-hydroxycorticosteroid concentrations while others have shown no eosinopenic response despite adequate elevations of 17-hydroxycorticosteroids. On the basis of these exceptions, the question is raised whether the eosinopenic response to ACTH *need* be mediated by an increased secretion of 17-hydroxycorticosteroids by the adrenal cortex.

In contrast to the generally adequate response of 17-hydroxycorticosteroids to ACTH, usually no elevation of these steroids occurs in response to epinephrine, despite an adequate eosinopenia. On the basis of these data there is a strong suggestion that the eosinopenia which occurs in response to epinephrine *is not mediated* by an increased circulating concentration of 17-hydroxycorticosteroids.

Summary. 1. A comparison of the responses of eosinophils and of circulating 17-hydroxycorticosteroid plasma concentrations is presented. 2. In general, responses of eosinophils and 17-hydroxycorticosteroids to the injection of ACTH are adequate, with a reduction of the former and an elevation of the latter. 3. Certain exceptions to this generalization are noted. On the basis of these exceptions the question is raised whether the eosinopenic response to ACTH need be mediated by the mechanism previously accepted. 4. There is a strong suggestion that the eosinopenia in response to epinephrine is not mediated by an increased circulating concentration of 17-hydroxycorticosteroids secreted by the adrenal cortex.

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Effect of a Series of Ethylenimine Derivatives against Metastasizing Mammary Adenocarcinoma of the Rat.   (19961)

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Two *N*-ethylene substituted phosphoramides were reported by Buckley *et al.*(1), Burchenal *et al.*(2) and Lewis *et al.*(3) to be as effective as Triethylene melamine in retarding the growth of a number of experimental tumors. The inhibitory effect of a large series of ethylenimine derivatives against a wide tumor spectrum has been reported independently by this laboratory(4,5).

The purpose of the studies reported here was to select ethylenimine derivatives* which markedly inhibit the metastasizing tendency of the mammary adenocarcinoma of the Hooded Brown Rat.

Materials and methods. Approximately 2 x 2 mm fragments of mammary adenocarcinoma, R2426, were implanted aseptically by trocar into the subcutaneous tissue of the right side of inbred Hooded rats weighing 100-120 g. The rats were weighed, housed individually and arranged 7 females and 8 males per group. Treatments were started 48 hours after implantation. Included in each experiment were one saline-treated group that served as a negative control, and one Triethylene-melamine-treated group that served as a positive control. A synthetic diet(5) and water were

given *ad libitum.* The compounds were prepared in buffered saline and the concentrations used as indicated in Table I. Injections were administered intraperitoneally daily or every other day in a volume of 0.5-1.0 ml. The period of treatment was approximately 42 days. At the end of this time all animals were sacrificed and the lungs excised. Observations were made on the gross metastatic lesions of the lungs and a section† was fixed for histological study. The inhibitory effects on the primary tumor have been reported previously(5).

Results. The pooled results of several experiments in Table I indicate that the 5 compounds at the levels tested markedly reduce the incidence of metastases to the lungs. Fig. 1 illustrates the effect of these compounds on the gross metastatic tumors of the lung. The control lungs on the left show the typical metastatic growths. On the right is shown typical treated lungs with no visible metastatic lesions. Histologically the control lungs show tumor masses so extensive that the normal architecture of the lungs has been nearly destroyed. The lungs of the treated animals

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* Supplied by Calco Chemical Division, American Cyanamid Co., Bound Brook, N. J.

† The authors are indebted to Mrs. Hester S. Schurr for the preparation of the tissues for histological study.

TABLE I. Effect of Phosphoramide Series on Lung Metastases.

Chemical No.	Chemical name	Treatment, mg/kg	No. T c̄ M / No. T. rats	No. K c̄ M / No. K rats	% T c̄ M / % K c̄ M
3155C	N,N′,N″-Triethylenephosphoramide	.25– .5	17/37	25/36	46/70
3172C	N,N′,N″-Tris(1-methylethylene)phosphoramide	2.5 –10	2/17	12/24	10/50
3173C	N-(3-Oxapentamethylene)-N′, N″-diethylenephosphoramide	1.75– 2.5	10/24	14/21	38/67
3193C	N,N′,N″-Triethylenethiophosphoramide	.5 – 1	6/24	17/23	25/74
3066C	Triethylene melamine	.1	11/49	35/57	18/58

K = Control.   M = Metastases.   T = Treated.

have only a few sparsely scattered viable tumor cells in close proximity to the larger blood vessels.

Fifty to 74% of the negative control rats developed gross metastases in the lungs 6.5 weeks after the implantation of the primary tumor. In the case of the rats treated with the ethylenimine derivatives, only 10-46% had

FIG. 1. Excised lung. Left row, untreated; right row, treated.

small gross metastatic lesions.

The chemicals greatly reduced the size of the primary tumors.

Discussion. All of the compounds of this series significantly affected the development of the metastatic foci in the lungs. The most potent one, unfortunately, is unstable. However two of the chemicals, N-(3-Oxapentamethylene)N′,N″ - diethylenephosphoramide (3173C) and N,N′,N″-Triethylenethiophosphoramide (3193C), are stable and compare favorably with compounds now showing clinical promise(6,7).

Summary. 1. A method has been developed for testing chemicals against the secondary metastatic foci of the mammary tumor. 2. Five compounds were found to be active in inhibiting the development of metastatic foci. N,N′,N″-Tris(1-methylethylene) phosphoramide and N,N′,N″-Triethylenethiophosphoramide were most active whereas the others are only moderately so.

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Combination Chemotherapy of Cancer: Potentiation of Carcinostatic Activity of 8-Azaguanine by 6-Formylpteridine.* (19962)

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(Introduced by David V. Habif.)

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In a previous publication(1), it was reported that combination therapy with folic acid and 8-azaguanine produced greater carcinostasis than did therapy using 8-azaguanine alone. Folic acid itself caused no inhibition of the growth of the 755 mouse breast carcinoma used in these experiments. Insight into the mechanism of action whereby the carcinostatic activity of 8-azaguanine is augmented by folic acid was obtained through the observation that mammalian tissues contain enzymes capable of deaminating 8-azaguanine to 8-azaxanthine(2-4). Unpublished experiments evaluating the latter compound against tumor growth revealed this deaminated product of 8-azaguanine to be inactive as a carcinostatic agent. These initial observations have recently been confirmed and extended (4).

It was therefore hypothesized that folic acid or its degradation product 2-amino-4-hydroxy-6-formylpteridine (6-formylpteridine) inhibited the enzymatic deamination of 8-azaguanine *in vivo*, preventing the conversion of this carcinostatic agent into the non-carcinostatic compound, 8-azaxanthine(1). *In vitro* demonstration of the inhibitory effect of folic acid upon the enzymatic deamination of guanine and 8-azaguanine has been obtained

(2). Evidence is presented in this report that (a) 6-formylpteridine augments the carcinostatic activity of 8-azaguanine, and (b) that this compound inhibits the enzymatic deamination of 8-azaguanine by tumor extracts.

Experimental. In vivo studies. The 755 tumor, a mammary adenocarcinoma, was subcutaneously transplanted to the axillary region of C_{57} black male mice by the usual trocar technic. The mice, weighing 18-25 g, and from 2 to 3 months of age received an *ad libitum* diet of Rockland pellets and water. The preparation of the 8-azaguanine for administration, its consistent carcinostatic effect upon the 755 tumor, the details of tumor growth measurement, and the method of statistical analysis have been previously described(1,5). 6-Formylpteridine, in weak alkaline solution, was injected intraperitoneally at various time intervals in relation to 8-azaguanine.

In vitro studies. The transplanted tumors (755) grown in C_{57} black mice, were removed immediately after killing the animals with ether, and stored for 24 or 48 hours in the frozen state until used. Pooled tumor tissue was homogenized for 3-5 minutes in a glass homogenizer of the Potter type with ice cold 0.1 M borate buffer at pH 8.4. The homogenates were then centrifuged at 22,000 g for 20 minutes in the cold. The resulting clear, pink, supernatant fluid at a concentration equivalent to 30% fresh tissue was used as the enzyme source. All operations were carried out at 4°C. 8-Azaguanine solutions were prepared as described previously(2) except that the

TABLE I. Effect of 6-Formylpteridine Alone and in Combination with 8-Azaguanine on the 755 Carcinoma in C57 Male Mice.

Exp. No.	Group	Dose,* mg/kg	Time interval, hr†	Mean tumor wt, mg	No. of animals, dead/total	% change in body wt
38	Aza.			2049	3/20	+ 6
	Aza. + 6–FP ⎰	20	Simult.	1614	1/20	— 5
	⎱	20	1	1661	4/20	— 6
	⎱	20	½	1245	2/19	— 7
56	Control			863	0/19	+11
	6–FP	20		833	2/20	—13
	Aza.			438	0/20	+ 1
	Aza. + 6–FP ⎰	20	Simult.	488	1/20	— 2
	⎱	20	½	230	0/20	— 5
	⎱	20	1	265	0/18	— 3
58	Aza.			321	0/17	+ 3
	Aza. + 6–FP ⎰	15	½	182	0/17	— 4
	⎱	20	½	225	0/17	— 6
	⎱	20	1	281	0/18	— 3
64	Aza.			737	0/19	+ 1
	Aza. + 6–FP ⎰	20	½	358	0/20	— 6
	⎱	10	½	563	0/20	— 2
	⎱	10	¼	596	0/20	— 2
66	Control			1712	1/10	+13
	6–FP	20		1390	2/10	+ 4

* Refers to dose of 6-Formylpteridine. 8-Azaguanine always inj. at a dose of 50 mg/kg.
† 6-Formylpteridine inj. prior to 8-Azaguanine.
Daily intraper. therapy was begun upon well established tumors varying between experiments from 4 to 7 days old. The duration of tumor growth varied between experiments from 21 to 27 days.

solvent used was 0.1 M borate buffer at pH 8.4. 6-Formylpteridine solutions were prepared similarly. The method used for following the deamination of 8-azaguanine by tumor extracts is based on a microdetermination of the ammonia nitrogen evolved during enzyme action (2,6).

Results and discussion. The *in vivo* effect of 6-formylpteridine alone and in combination with 8-azaguanine upon the 755 adenocarcinoma grown in the C_{57} male mice is recorded in Table I. Administered alone, 6-formylpteridine had no influence upon the growth of the neoplasm. The results obtained by the combination of 6-formylpteridine and 8-azaguanine depended upon the time interval between the injection of these 2 drugs. Negative results were consistently obtained if the 2 compounds were administered simultaneously. Statistically significant augmentation of the carcinostatic activity of 8-azaguanine occurred when the 6-formylpteridine was given a half hour prior to the injection of 8-azaguanine. Variable results, ranging from negative to almost significant enhancement of the

inhibitory effect of 8-azaguanine upon tumor growth, ensued when the time interval was extended to one hour. Dosages of 6-formylpteridine less than 15 mg per kg body weight produced negative results regardless of the time interval. Only mild toxicity evidenced

TABLE II. *In Vitro* Inhibition of Tumor Guanase by 6-Formylpteridine.*

6-Formylpteridine moles/ml reaction mixture	μg NH_2-N/g tissue wet wt present after 2 hr incubation with substrate at 37°C†	
	Pre-incubated‡	No pre-incubation§
.000	336	
.001	227	
.006	128	
.011	68	261
.016	41	

* 755 mammary adenocarcinoma grown in C57 mice. 8-Azaguanine was added in all cases to a level of .011 mole/ml of reaction mixture. Each reaction vessel contained ≅ 300 mg tissue, wet wt.
† Corrected for endogenous ammonia.
‡ Enzyme incubated 30 min. with 6-Formylpteridine before addition of 8-Azaguanine.
§ 6-Formylpteridine and 8-Azaguanine added simultaneously.
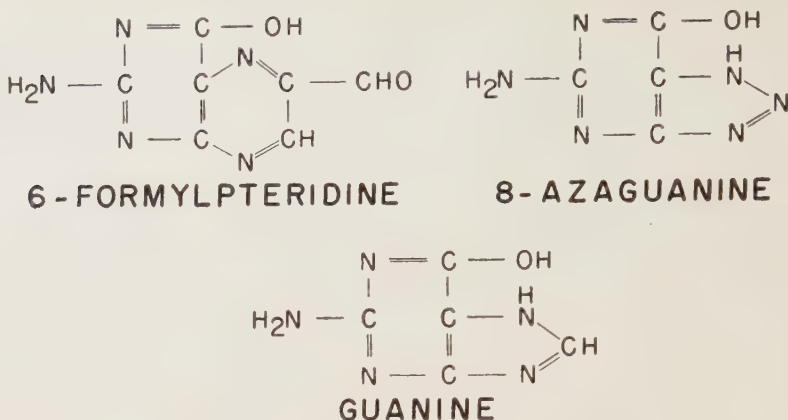
FIG. 1.
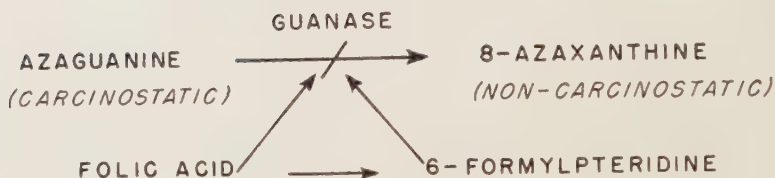
FIG. 2.

by slight loss in weight was observed in the group of animals receiving the drug combination.

6-Formylpteridine was observed to inhibit the *in vitro* deamination of 8-azaguanine by tumor extracts (Table II). This effect was most evident when the 6-formylpteridine and tissue extracts were incubated together for a 30-minute period prior to the addition of the 8-azaguanine. Simultaneous addition of inhibitor and substrate produced only slight inhibition. It is of interest that 6-formylpteridine, the primary photolytic decomposition product of folic acid(7,8), although structurally similar to both 8-azaguanine and guanine (Fig. 1), is itself not deaminated.

The lag in inhibition observed when 6-formylpteridine and 8-azaguanine were added simultaneously is of prime importance since it would seem to give insight into the *in vivo* phenomenon regarding the time interval necessary between 6-formylpteridine and subsequent 8-azaguanine administration for optimum augmentation of the carcinostatic effect of the latter compound. That a time interval is essential *in vitro* as well as *in vivo* tends to indicate that the observed effect is due either to a slow combination of the enzyme with inhibitor or to a further enzymatic modification of the molecule to one which is the true inhibitor. It seems unlikely that the necessity for a time interval is explained on the basis of differential tissue absorption of the 2 drugs.

The *in vitro* studies presented, although preliminary in nature, indicate that 6-formylpteridine can act as an inhibitor of 8-azaguanine deamination, thereby blocking the detoxification of 8-azaguanine and leaving more of this carcinostatic agent available at the tumor site (Fig. 2). The *in vivo* data demonstrating potentiation of the carcinostatic activity of 8-azaguanine by 6-formylpteridine supports this explanation. Such an hypothesis is given additional support by recent work demonstrating that differences in tumor guanase activity may account for the susceptibility or non-susceptibility of tumors to inhibition by 8-azaguanine(4).

These observations emphasize the importance of time-dosage studies not only in regard to fundamental mechanism studies, but also in the more practical application of combination chemotherapy.

Summary. 1. 2-Amino-4-hydroxy-6-formyl-

pteridine (6-formylpteridine), although non-carcinostatic by itself, augmented the carcinostatic action of 8-azaguanine against a mammary adenocarcinoma. This effect was found to be dependent upon a time interval between the injection of 6-formylpteridine and 8-azaguanine. 2. 6-Formylpteridine inhibited the *in vitro* deamination of 8-azaguanine by tumor extracts. Pre-incubation of enzyme with inhibitor was found to be essential for maximal inhibition. 3. It was postulated that the enhanced carcinostatic effect observed *in vivo* could be due to inhibition of 8-azaguanine deamination by 6-formylpteridine, since the deaminated product (8-azaxanthine) is non-carcinostatic.

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Influence of Cortisone on Nucleic Acids and Protein Content of the Chick Embryo.   (19963)

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It has been demonstrated by Karnofsky *et al.*(1) that cortisone inhibits both the somatic development and the growth of the chick embryo. In view of the relationship existing between nucleic acid metabolism, protein synthesis and growth, it seemed interesting to study whether this inhibitory effect of cortisone on growth is associated with some change in the ribonucleic (RNA), desoxyribonucleic (DNA) acids and in the protein nitrogen (N) content of the chick embryo.

Methods. Fertile White Leghorn eggs purchased on the market were incubated at $38°C$ and 75% relative humidity. At 8 days of development, the chorioallantoic membrane was injected with 1.25 mg of cortisone acetate (Merck) per egg; controls were similarly treated with the saline suspension alone. The surviving embryos were sacrificed starting from the 11th to the 18th day of incubation, *i.e.*, from 3 to 10 days after the treatment, 4 embryos per group per day. They were weighed after freezing, then transferred to a Waring Blendor. The homogenate was analyzed for protein N content (precipitation with 14% trichloracetic acid and micro-Kjeldahl determination) and for DNA and RNA content by the method of Schmidt-Thannhauser(2).

Results. In the cortisone-treated embryos, growth and somatic differentiation were clearly inhibited starting from the third day after hormone injection; the effect being more evident in the following days. Fig. 1 presents the mean body weight and the analytical data in control and cortisone treated embryos; the protein N and the DNA and RNA values are plotted as mg of N and as μg of P, respectively. It appears that corti-
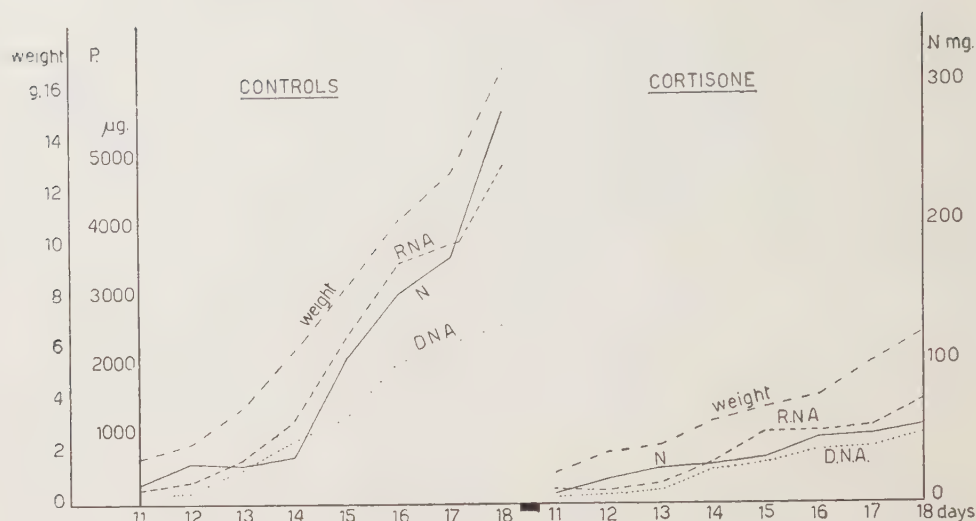
FIG. 1. Effect of cortisone treatment on wt and some biochemical constituents of the whole chick embryo.

sone definitely inhibited the growth and lowered the protein and nucleic acid content of the whole chick embryo. In order to investigate further the phenomenon, the mean analytical values of the embryos over the entire period of study have been calculated and referred to 100 g of wet weight (Table I). Cortisone treatment produced a significant percentage decrease in protein and RNA, while the DNA percentage was practically unchanged.

Discussion. The inhibition by cortisone of the growth of the chick embryo parallels the similar effect of the hormone on the growing and adult rat, first demonstrated by Wells and Kendall(3) and recently emphasized by

TABLE I. Mean Values per 100 g Wet Body Weight of Controls and Cortisone-Treated Chick Embryos.

	Controls			Cortisone		
	N, g	DNA, mg P	RNA, mg P	N, g	DNA, mg P	RNA, mg P
Mean	1.19	13.9	23.4	.95	12.9	18.51
S.E.*	.08	.83	.96	.07	.91	1.33
P†				<.05	×	<.01

* Stand. error $= \sqrt{\dfrac{\Sigma \cdot d^2}{n(n-1)}}$.

† Calculated according to the test of Student.

Cavallero *et al.*(4). Moreover, from the present data it appears that inhibition of growth is associated with a definite decrease of protein and RNA content of the body, but not of the DNA. Since this decrease is evident when the values are referred to unit weight, it appears to be independent of the inhibition of weight.

Baker and Ingle(5) and Lowe *et al.*(6) have shown that under the influence of ACTH or cortisone, the ribonucleic acid and protein nitrogen concentrations of liver cells are diminished, and Kass and Kendrick(7) have made similar observations on the lymph nodes of rats treated with cortisone. From the above, it seems likely that cortisone influences the RNA metabolism and the protein synthesis of the cells; therefore the inhibition of the growing organism by cortisone appears to be related to some disturbance of protein synthesis and is closely correlated with a decrease of the RNA content of the organism.

Summary. Cortisone inhibited the growth and concurrently reduced the protein, the DNA and the RNA content of the whole chick embryo; when referred to the unit weight, only the protein and the RNA showed significant decreases. It is suggested that the growth inhibiting effect of the hormone is closely connected with a decrease of protein

and RNA synthesis.

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Poliomyelitis and Coxsackie Viruses Isolated from Normal Infants in Egypt.* (19964)

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In Egypt, poliomyelitis is most unusual in native adults and has been rarely reported at any age. Yet, as Paul(1,2) has pointed out, the endemic paralytic attack rate for this disease in Egyptian infants is appreciable. Furthermore, a serological survey carried out on "normal" sera collected in Egypt in 1950, revealed that neutralizing antibodies to all three types of poliomyelitis virus are also acquired at an early age among native Egyptians. For type 2, more than 50% of infants have antibodies before they are 2 years of age, and for all 3 types 75 per cent or more before they are 4 years old. Complement fixing antibodies, which are transient in poliomyelitis, were also measured in this population(3), and the results supported the view that in Egypt infection with poliomyelitis virus must be widespread in the early years of life.

To extend these studies, 36 rectal swabs were collected in Egypt‡ from normal babies between 6 and 12 months of age, *i.e.*, at a time in life when the studies referred to above indicated maximum prevalence of infection. The purpose here was to determine how frequently one might detect poliomyelitis virus (and other viruses) in the intestinal tract of normal infants. The samples were collected by Dr. Robert Ward, while in Egypt carrying out a study of the occurrence of poliomyelitis virus in flies(4), and were generously supplied to us. The swabs were tested not only for poliomyelitis virus, but for Coxsackie (C) viruses as well, and both viruses were found.

Collection of specimens. Specimens were collected only from well children between 6 and 12 months of age, who were inhabitants of villages in the neighborhood of Cairo, representing an area under study by the International Health Division of the Rockefeller Foundation. It was in this same district that specimens for the serological studies mentioned above(2,3) had been collected. The swabs were placed in 1 ml of sterile water and frozen on dry ice. They were transported in the frozen state to New Haven where the laboratory studies were carried out. Sera from the children were also obtained, frozen soon after collection, and also transported on dry ice.

Methods. After thawing, the fluid contained in each swab was expressed by placing the swab in the barrel of a 1 ml syringe and forcefully squeezing it with the piston. The expressed fluid was added to the fluid in which the swab had been soaked, a total of about

TABLE I. Isolation of Viruses from Egyptian Infants.

Pool	Poliomyelitis virus	Coxsackie virus	Tissue culture agents*
A	—	—	—
B	—	+	+†
C	—	+	—
D	+	+	—
E	—	—	—
F	—	+	+*
G	—	+	—
H	—	+	—
I	—	—	—
J	—	+	—
Total	1/10	7/10‡	2/10

* On passage in tissue culture this agent became markedly cytopathogenic for monkey testicular fibroblasts. It remained nonpathogenic *in vivo* for mice (infants and adults), hamsters, and monkeys, and has not yet been identified.

† This strain produced mild fibroblastic degeneration in monkey testicular cultures. Tissue culture fluids produced paralysis with myositis in infant mice typical of Coxsackie viruses. The strain was subsequently identified as belonging to the Boston type(8).

‡ The 7 positive pools contained 26 individual samples. When these were run separately, 9 yielded positive tests of C virus, 5 gave incomplete tests, and 12 were negative.

1 ml being available from each specimen. The fluids were centrifuged for 15 minutes at 18,000 rpm in the PR-1 refrigerated International centrifuge and the supernate saved. The 36 rectal specimens were made into pools, each containing material from 3 to 4 swabs. Before inoculation, a mixture of penicillin and streptomycin was added so that the inoculum contained 1000 units of each per ml. The pools were tested intracerebrally in monkeys for poliomyelitis virus and subcutaneously in 1-day-old mice for C viruses. In addition the specimens were tested in roller tube cultures of monkey testicular tissue according to the methods previously described(5). Sera from the same 36 infants were each tested, in infant mice for the presence of C viruses, and in both infant and young adult mice for the presence of encephalitis viruses. The latter tests were run in view of the previous isolation of 3 strains of West Nile virus from "normal" sera collected in this area in 1950(6).

Results. As shown in Table I, one strain of poliomyelitis virus, 9 of Coxsackie virus, and an unknown agent were isolated from these normal children.

Poliomyelitis virus as detected by monkey inoculation. Following intracerebral inoculation in monkeys, the 10 pools yielded one, D, which was positive for poliomyelitis virus. When tested for cytopathogenicity in roller tube cultures of monkey testicular tissue, this sample was negative.

Coxsackie viruses as detected by mouse inoculation. The 7 pools which were positive for C viruses produced paralysis in infant mice 3 to 9 days after inoculation. With several of the samples, all inoculated infant mice succumbed. Lesions were typical(7) and limited to the skeletal muscle. The individual components of the 10 pools were then rethawed and run separately. Some of the pools contained more than one positive specimen; the 7 positive pools were made up of a total of 26 samples, and of these, 9 samples were positive, 5 gave incomplete tests (all mice died without signs of paralysis having been observed), and 12 were negative. Seven of the Coxsackie strains were typed(8): 2 belonged to Israel-7 type, 2 to Texas-1, 1 to Texas-15, 1 to Boston, and 1 to Type 3.

Tissue culture studies. Of the 10 pools, two, B and F, produced cytopathic changes (fibroblastic degeneration) such as have been previously described for Coxsackie and poliomyelitis viruses, respectively, in monkey testicular tube cultures(5). The agents were passed serially through 5 tissue transfers including 15 changes of the fluid medium. Pools B and F had yielded positive tests for Coxsackie virus on direct inoculation into infant mice, and negative tests for poliomyelitis virus in monkeys. The members of the 2 pools were then run separately.

In monkeys, the 4 components of pool B and the 3 components of pool F each gave negative tests for poliomyelitis virus. In mice each pool yielded one sample positive for C virus.

The two agents isolated in tissue culture from pools B and F were tested in infant mice after 3 *in vitro* passages. The F agent was negative in 32 mice. On the other hand, the B agent produced paralysis in 33 mice, 18 of which had been inoculated intracerebrally and 15 subcutaneously. Mice developed signs of disease on the 5th or 6th day, and histological sections of 25 animals revealed typical

Coxsackie lesions in the muscles, with none present in the brain, spinal cord, or fat.§ After 2 passages in mice, the virus was titrated and yielded a LD$_{50}$ of 10$^{-6.0}$. It was identified as belonging to the Boston type of Coxsackie virus both by neutralization and complement fixation tests(8).

In tissue culture, the agent isolated from pool F produced definite fibroblastic degeneration by the 5th day which progressed to marked degeneration by the 9th day. It readily passed through an EK Seitz filter. Fluids harvested on the 5th and 9th days after inoculation of the culture tubes failed to produce disease or CNS lesions in 2 cynomolgus and 3 rhesus monkeys inoculated intracerebrally. The agent remained nonpathogenic for mice, both newborn and adult, and also for hamsters. Neutralization tests were carried out in tissue culture(5) using potent antisera to Types 1, 2, and 3 poliomyelitis virus, and none of the sera were able to inhibit the cytopathogenic effect of the agent. Obviously this filterable agent requires more study.

The sera of all 36 children failed to produce disease when tested individually by intracerebral inoculation into 4-week-old Swiss mice (8 mice for each serum) and by intracerebral and subcutaneous inoculation into newborn mice (8 to 16 mice for each serum), indicating that they were free of lethal agents such as West Nile and Coxsackie viruses.

Comment. These findings give additional support to the concept that poliomyelitis is an endemic disease of the tropics, and they supplement earlier studies(2,3) indicating that infection with poliomyelitis virus may occur at a relatively early age in Egypt. In addition to the previous serological evidence, we can now record the isolation, during a non-epidemic time, of poliomyelitis virus from the intestinal tract of a supposedly normal infant in the area, representing one of the 36 infants tested. This observation is similar to that of Gear *et al.*(9) who found 4 of 16 native infants in the Johannesburg area excreting virus at some period during the first year of life. As only one rectal swab was collected

§ We are indebted to Dr. E. Manuelides for preparing and examining these sections.

from each child in the present study, this single positive among the 10 pools is of significance. Further evidence for the widespread prevalence of poliomyelitis virus in the Cairo area is indicated by the ease with which Ward has been able to detect virus in flies trapped there (4).

Within the last year or so, C viruses have been isolated in the Middle East from patients (10), from Cairo sewage(11), and from flies (4). A variety of types have been identified from these sources(8). The present data indicate how common infection with these viruses may be in this area, which is similar to the experience of Measroch *et al.* who found that 5 of 9 native South African babies tested were excreting C viruses in their feces(12). Only one of their babies had an illness at the time of the collection of the specimens from which virus was isolated.

One of the C viruses described in the present study (pool B) is characterized by its cytopathogenic property in tissue culture. As few C viruses have this property, it is noteworthy that this one is antigenically related to the Boston type (Wieder strain) which has been found to produce cytopathic changes in roller tube cultures of human(13) and monkey tissues(5).

The isolation of a filterable agent, as yet unidentified, depended upon the use of the new tissue culture technic(13). Like similar agents recently isolated in the United States (5), the Egyptian agent is characterized by its cytopathogenicity for fibroblasts in monkey testicular cultures, and its avirulence for monkeys, mice, and hamsters. Antisera to the three poliomyelitis virus types had no inhibitory influence on it. The role of this agent in human disease is completely unknown. Somewhat similar agents cytopathogenic for human tissue cultures have also been isolated in Boston(13). The relationship of the Egyptian agent to American ones with pathogenicity similarly limited to tissue culture remains for further study.

Summary. Thirty-six rectal swabbings from normal Egyptian infants, tested singly or in pools representing material from 3 to 4 infants yielded 1 poliomyelitis virus, 9 Coxsackie (C) viruses, and 1 unidentified filterable agent.

The unidentified agent was characterized by its cytopathogenicity for cultures of monkey testicular tissue and by its failure to produce disease in monkeys, mice, and hamsters.

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Epidemic Influenza B and C in Navy Recruits during Winter of 1951-52.
(19965)

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Recognition has recently been given to a strain of virus classified as "Influenza C" (1,2) but multiple isolations of strains of this type from a population during a single winter has not been previously reported.[†]   Fourteen viruses, serologically related to earlier strains of influenza C, were isolated from Navy recruits between January and April of 1952. Evidence that these viruses were epidemic should be of interest to others concerned with the etiology and epidemiology of influenza during the past winter. Epidemic influenza B occurred simultaneously in this population. Eleven influenza B viruses were isolated.

Materials and methods. Observations on influenza have been made among recruits at the Naval Training Center, Great Lakes, during each winter since 1948. Continuing these observations during November, 1951, and June, 1952, throat swabs for virus isolations and acute and convalescent sera were routinely obtained from Navy recruits hospitalized because of acute respiratory infections including suspected cases of influenza and cases of pneumonia. Paired sera also were available from serial bleedings done at various intervals on 13 companies of recruits undergoing training between January and May. Acute and convalescent sera from patients experiencing

† A few were identified at the University of Michigan during March 1952(5).

TABLE I. Fourfold or Greater Rises in Hemagglutination-Inhibition (H-I) Antibody Titer Against Influenza Viruses PR-8, RM-1, or LEE, and Isolations of Influenza A and B Viruses in Navy Recruits Hospitalized with Acute Respiratory Disease.

| Period (1951-52) | No. pairs sera completed | Patient's sera No. 4-fold rises in H-I titer to: | | Throat swabs or nasal washings No. of specimens | No. of viruses isolated | | Resp. dis. admissions | |
		A or A′	B		A	B	No.	Rate*
11/11–12/15	181	2	1	116	0	0	236	69
12/16– 1/12	98	1	1	127	0	1	173	52
1/13– 2/9	252	10	30	135	0	3	525	109
2/10– 3/8	238	3	4	102	0	3	464	101
3/9 – 4/5	137	0	6	136	0	3	197	53
4/6 – 5/3	20	1	0	70	0	1	147	48
5/4 – 5/31	22	0	0	18	0	0	159	55
Total	948	17	42	704	0	11	1901	—

* Rate/1000 recruits/period.

influenza-like illnesses during the winter were also supplied from other localities‡ (Table IV).

Nasal washings were collected in 30 ml, and throat swabs were immediately placed in 2 to 3 ml of nutrient broth. Routinely, 1000 units of penicillin and 1 mg of streptomycin were added per ml of filtered nasal washing or throat swab suspension prior to intraamniotic inoculation of 13-day-old embryonated eggs. After incubation for 3 to 4 days at 35°C, the amniotic fluid was harvested and tested for the capacity to agglutinate chicken and guinea pig erythrocytes after being left at 4°C for 60 to 90 min. At least 2 egg passages were made before any specimen was called negative. Salk's modification(3) of the Hirst hemagglutination-inhibition test was employed in the identification of newly isolated viruses, using rooster immune sera§ and 4 agglutinating units of virus. Antibody titrations on sera followed the hemagglutination-inhibition technic of Hirst and Pickels(4).

Observations on influenza among recruits hospitalized with acute respiratory diseases. Data relating to the occurrence of influenza A or B in patients with acute respiratory diseases are summarized in Table I. Admissions include all types of acute respiratory infec-tions hospitalized. A large proportion of the patients in January and February had strepto-coccal infections.‖ There was little evidence of epidemic influenza as manifested by ex-cessive numbers of admissions for clinically diagnosed influenza at any time during the winter despite the laboratory evidence for the occurrence of this disease. Eight of the 11 influenza B strains were isolated from patients clinically diagnosed as influenza, 2 were from patients diagnosed as acute pharyngitis, and one was from a patient with pneumonia of undetermined etiology. The influenza B strains isolated were immunologically related to the 1210 strain. Fourteen viruses, identi-fied as antigenically similar to the JJ strain of influenza C virus(2), were also isolated from recruits hospitalized because of acute respiratory diseases. Nine of the patients had influenza-like illnesses, 2 were diagnosed as acute pharyngitis, and 2 had pneumonia of undetermined etiology. As in the case of in-fluenza B, there appeared to be nothing dis-tinctive about these illnesses.

The dates of isolation and immunological characterizations of the strains identified as influenza C are given in Table II. Also shown are the antibody titers to the homologous strain isolated from each patient in the pa-tient's acute and convalescent sera. One in-fluenza B strain (GL 704B) and influenza C strain (GL 899C) were selected as prototypes

‡ The Student Health Clinic of the University of Chicago, Fort Ord, Calif., (Dr. E. H. Lennette), Com-municable Disease Center, Montgomery, Ala., and the National Institute of Health (Dr. Joseph Bell).

§ The JJ strain of influenza C used in preparation of immune rooster sera was kindly given us by Dr. Thomas Francis, Jr.

‖ Oral penicillin prophylaxis for streptococcal dis-ease was given to a majority of recruits during March, 1952.

TABLE II. Hemagglutination-Inhibition (H-I) Titrations for 14 Strains of Influenza C Viruses Using Rooster Immune Sera for A, B, and C (JJ) Strains of Influenza Virus and Patient's Acute and Convalescent Sera with His Homologous Strain of Virus. Titers are expressed as the reciprocal of dilution of sera.

Date of isolation, 1952	Strain	Rooster immune sera				Patient's homologous sera	
		FM-1	Lee	1210	JJ	Acute	Convalescent
Jan. 22	GL 585				1280	64	64
22	596				1280	20	160
Feb. 7	695				640	256	1024
14	756				1280	32	32
18	779				2560	32	32
19	782				640	32	32
26	832	<20	<20	<20	2560	32	1024
26	833				1280	128	1024
Mar. 10	899				1280	40	640
12	919				2560	32	64
12	920				2560	128	128
Apr. 23	1082				1280	32	512
24	1090				1280	128	256
24	1091				2560	64	64

for use in serological studies. The GL 704B strain was isolated from the nasal washings of a recruit during an influenza-like illness on February 8, 1952. The GL 899C strain was isolated from the nasal washings of a recruit ill with pneumonia of unidentified etiology on March 10, 1952.

Relation of influenza B and C to acute respiratory infections in humans. Clinical and bacteriological data collected weekly and sera from 5 bleedings were available for analysis on a company of 82 recruits who entered training on January 15, and departed on April 16, 1952. Hemagglutination-inhibition tests with the PR 8, FM 1, and Lee strains of influenza virus showed that 19% of the men had significant antibody rises to these strains during this period of time. The serial sera of 25 men in this company were picked at random and

also analyzed for antibody rise to the GL 899C strain of influenza C virus and to the GL 704B strain of influenza B virus. A 4-fold or greater rise in titer against these viruses was considered to indicate an infection with the virus when all sera from one man were run in a single test. The results are shown in Table III. Twelve individuals showed 4-fold or greater rises in antibody titer to the GL 899C antigen while 7 showed similar rises to the GL 704B strain. Antibody rises to both viruses occurred in 4 men and in only 2 were these rises concurrent. A comparison with previous data (Table I) showed that there was no correlation between rises to the influenza C virus and rises to the PR 8, FM 1 or the Lee antigens. Of the 12 men showing antibody rises to influenza C virus, 6 could be correlated with a minor respiratory illness clinically classified as the common cold (five) or influenza (one), whereas only one of 7 individuals showing rises to influenza B virus had a correlating respiratory tract illness. Minor respiratory infections and influenza-like illness which did not provoke a rise in antibody titer to the influenza antigens also occurred frequently in the 25 men. These data would suggest, however, that the influenza C virus may have been related to a minor respiratory illness, whereas influenza B virus produced symptoms less frequently.

TABLE III. Hemagglutination-Inhibition (H-I) Antibody Increases to the GL 899 Strain of Influenza C and the GL 704 Strain of Influenza B in Serial Sera Obtained from 25 Navy Recruits Between January and April, 1952.

Period of H-I rise	4-fold rises in H-I titer to	
	GL899C	GL704B
1/15–1/31	4	1
1/31–2/14	2	5
2/14–3/31	6	1
3/31–4/16	0	0
Total	12	7

TABLE IV. Hemagglutination-Inhibition Titers to GL 899 Strain of Influenza C Virus in Paired Sera Obtained from Patients with Influenza Like Illness in Other Areas of United States and in Sera of Navy Recruits from Which This Strain Was Isolated.

Source of sera	No. pairs tested	Titers of initial sera							No. 4-fold rises
		32	32	64	128	256	512	1024	
CDC,* Ala.	10				1	2	5	2	0
Ft. Ord., Calif.	20	1	4	4	1	6	4		1
U. Chicago, Ill.	30		1	6	14	8	1		3
Great Lakes, Ill.	14	1	6	3	3	1			6

* Communicable Disease Center.

Other serological studies on influenza A and B among recruits. Sera obtained at the beginning and the end of training from 370 recruits from 5 companies in residence between November and May were tested for antibody rises to influenza A and B virus antigens. Fourfold increases in hemagglutination-inhibition antibody titer for either the PR 8 or FM 1 influenza viruses occurred in 4% and for the Lee influenza virus, in 14% of these men.

Other serological studies of influenza C among recruits. Six pairs of sera were selected at random from each of a total of 12 companies of recruits from which sera were available. Of these 72 pairs of sera, 11 or 15%, showed a 4-fold rise in hemagglutination-inhibition titer to the GL 899 strain of influenza C virus.

Influenza C elsewhere. Sera obtained elsewhere were also tested with the GL 899C strain and the results are shown in Table IV. Three pairs from the National Institute of Health were also run and showed no rise. For comparison, the titers in the sera of Navy recruits from whom the virus was isolated are included in Table IV. Four-fold rises occurred in 3 of 30 pairs of sera from the University of Chicago and in one of 20 from Fort Ord, California, indicating that the virus may be widespread in the population.

Biological characteristics of influenza C viruses. The presence of the virus was always detected in the first or second egg passage by the capacity of the amniotic fluid to agglutinate chicken erythrocytes at 4°C. Serial amniotic passages of the GL 899C strain have been successful through 21 passages. The hemagglutination titers range from 512 to 2048 and the egg infectivity was 10^{-8} at the 21st passage. Several efforts have been made to adapt the virus to the allantoic membrane. Varying the age of the chick embryo, the length of incubation, and the concentration of the inoculum has seemed to have little effect on the virus growth obtained. The results have been inconsistent and only occasional titers as high as 1:128 have been obtained. Attempts at serial allantoic passage have failed. The virus agglutinated chicken erythrocytes at 4°C only. Neither guinea pig nor human type 0 erythrocytes were agglutinated. The hemagglutination titer of infected amniotic fluid decreased rapidly upon storage at 4°C, making it impossible to store the virus for serological tests at this temperature. The titer was maintained when stored in sealed ampules at −70°C. Two attempts were made to adapt the virus to Swiss mice weighing 12 g by intranasal inoculation. No lesions were observed in the lungs during 6 blind passages nor was there any virus detectable in mouse-egg-mouse passages.

Discussion. The experience with influenza B at Great Lakes is similar to that reported from many other parts of the United States during the winter of 1951-52. Patients from whom viruses were recovered had relatively mild clinical illnesses and developed antibody increases to the epidemic B strain and less regularly to the Lee strain. Of special interest also was the isolation of several strains of influenza virus type C from individuals showing similar illnesses. The routine performance of the hemagglutination test at 4°C in isolation and typing procedures was essential for the recovery of the influenza C viruses.

The recovery of 14 influenza C viruses and the immunological evidence of their prevalence during a period when influenza B was also prevalent in the same population is of interest since the isolation of both the JJ and the 1233 strains occurred during A and A′ epi-

demics. This serves further to set influenza C apart from influenza A or B as a distinct entity. Studies on the biological and immunological characteristics of the GL 899C strain of influenza C indicate its close resemblance to the JJ strain of Francis(2). Its relationship to the 1233 strain of Taylor(1) is under study.

Neither influenza B nor C viruses isolated at Great Lakes during 1952 seemed to possess marked virulence as only a small fraction of men showing diagnostic antibody rises had clinical illness of any degree of severity. However, in view of the past experience with influenza A and B, it is conceivable that the pathogenicity of the influenza C virus may change in subsequent years or in different localities. Thus, it would appear justifiable to look for this influenza virus strain with other etiological agents when investigating epidemic respiratory disease.

Summary. Data have been presented on the occurrence of influenza in Navy recruits at Great Lakes Naval Training Center during the winter of 1951-52. Serological evidence of occurrence of epidemic influenza B was confirmed by the isolation of 11 viruses which appear to resemble the 1210 strain of influenza B. The isolation of 14 strains characterized as influenza C and the serological evidence that influenza C was also epidemic are presented. One of these viruses, the GL 899C strain, was studied in more detail. Its biological and immunological properties and its relation to previously isolated strains were studied. Data were also presented indicating that influenza C infections occurred in other localities throughout the United States. The importance of performing routinely the CRC agglutination and CRC agglutination-inhibition tests at 4°C for the detection of influenza C viruses and antibody titer is stressed.

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Electrophoretic Studies on Cerebrospinal Fluid.* (19966)

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In a previous publication(1), a component (X-protein), migrating faster than albumin, was found to be a normal constituent of cerebrospinal fluid. This component has also been observed in a few cases by earlier workers(2,3). Labhart, Schweizer and Staub(4) analyzed 178 cerebrospinal fluids of healthy and diseased individuals with the aid of the Jamin interferometric optical system and failed to describe the presence of the rapidly migrating component. Recently Bücher, Matzelt and Pette(5), using paper strip electrophoresis, noted a rapidly migrating component in each of 10 cerebrospinal fluids which were classified as "normal". In the present paper, cerebrospinal fluids of patients with neurological disturbances were analyzed in the Tiselius apparatus in order to study the distribution of the components, particularly those migrating faster than albumin.

Method. Cerebrospinal fluids (50-220 ml) were obtained from patients undergoing pneumoencephalograms for diagnosis. Each fluid was centrifuged to remove cells and concentrated by pressure dialysis to final volumes varying from 3 to 5 ml. The sample was dialyzed against phosphate buffer of pH 7.85

TABLE I. Determination of γ-Globulin in Cerebrospinal Fluid by Elimination of δ-Boundary.

| | | Planimeter units | | | |
		$\gamma + \delta$ or ϵ	Other components	Total	$\gamma + \delta$ or ϵ fraction of total area
Control*	Asc.	67, 77	205, 257	298, 334	.225, .230
	Desc.	26, 27	264, 308	290, 335	.099, .081
12% H_2O	Asc.	42	264	306	.137
	Desc.	17	255	272	.063
14% H_2O beginning convections	Asc.	22	255	277	.079
	Desc.	16	250	266	.060
16% H_2O	Asc.	21	242	263	.080
	Desc.	12	235	247	.049

| | | Calculated (from ascending limb) γ-globulin | |
		(I) †	(II) †
Control		.04, .04	.12, .11

* Total protein conc., 1.15 g %.

† Calculated from equations: (I) $x = c.f.$; (II) $x^2 - x + \dfrac{A_A \cdot c.f.}{A_t} = 0$, where x is the size of the δ-boundary expressed as the fraction of the total area, A_A and A_t are the areas for the albumin and total pattern respectively, c is the protein concentration in g % and f is the size of the δ-boundary for a 1% albumin solution, expressed as the fraction of the total area.

and ionic strength 0.05. The protein concentrations of these samples, estimated with an Abbe refractometer, varied between 0.4 and 1.3 g %. The concentrated fluids were subjected to electrophoresis in a micro cell of 1.5 x 15 mm cross section and 50 mm height for 45 minutes at 10 volts/cm in a Klett model of the Tiselius apparatus, which was equipped with the Longsworth schlieren scanning mechanism. The ascending patterns only were evaluated and the components were classified in the same manner as are those of plasma.

Results. Interpretation of patterns. It has been shown previously that boundary anomalies may be utilized for demonstrating and separating rapidly migrating trace components (6). These anomalies are pronounced in respect to the albumin and the faster components in a phosphate buffer at pH 7.85 and ionic strength of 0.05 and they cause the apparent concentrations of the faster components to increase at the expense of the slower components(7,8). This phenomenon accounts for the present success in demonstrating at least one fast migrating component in every cerebrospinal fluid, in contrast to the results obtained with other buffers by earlier workers (2,3).

The cerebrospinal fluid patterns could not be analyzed by the standard procedure because the γ-globulin did not separate from the salt boundaries. The values for γ-globulin were obtained by subtracting the values for the δ-boundary which could be bracketed between two possible limits. These limits were obtained by assuming that, I) all proteins produce as large a δ-boundary as would be given by the same concentration of albumin, or II) the albumin alone contributes to the δ-boundary. The relative size of the area of the δ-boundary was determined for 4 bovine albumin (Armour) solutions, varying

TABLE II. Apparent Concentrations of Components at Different Protein Concentrations (% of Total Area).

| | Total protein conc. (g %) | | | | | | | |
| | .50 | | .67 | | 1.1 | | 2 | | |
	I	II	I	II	I	II	I	II	III
X	5	5	8	7	8	8	9	9	9
A	58	56	58	54	66	60	73	73	70
α_2	9	9	12	11	9	8	7	6	6
β	9	9	11	11	14	13	11	11	10
γ	19	21	11	17	3	11	0	1†	5*

* γ-globulin separated from δ-boundary; δ-boundary measured directly.

† If the error in the total protein were 10%, the value for the γ-globulin would be raised to 5%.

TABLE III. Comparison of Analyses of Cerebrospinal Fluids in Phosphate pH 7.8, $\Gamma/2$, .05 and in Veronal pH 8.5, $\Gamma/2$, 0.1.

Total protein, mg %	Buffer	Protein conc. in cell, g %	Limb	X I	X II	A I	A II	a_1	a_2 I	a_2 II	β I	β II	τ I	τ II	γ I	γ II
400	P*	1	Asc.	3	3	53	48		(a)		6	5	4	4	34	40
	V	1.40	{ Asc.	0		56.3		2.3	2.6		4		(b)		34.8	
			{ Desc.	0		56		2.8	2.5		5.6		(b)		33.1	
36	P	1.36	Asc.	16	14	60	50		9	7	11	9	4	4	0	16
		.55	Asc.	11	10	56	54		10	9	10	9	4	4	9	14
	V	1.	{ Asc.	2.8		55.4		8	9.2		20.4		(b)		4	
			{ Desc.					Not evaluated								
35	P	.55	Asc.	9	9	68	67		9	9	11	10	3	3	0	2
	V	.8	{ Asc.	4.1		55.8		7.7	9.8		14.4		(c)		8.2	
			{ Desc.	2.8		63.7		8.4	9.5		10		(c)		5.6	

(a) Low concentration of a_2-globulin, included in β-area.
(b)   ''   ''   '' τ-component,   ''   '' γ- '' .
(c) Not resolved.
* P = Phosphate; V = Veronal.

in concentration between 0.40 and 1.25 g %. A plot of the value for the fraction of the total area occupied by the δ-boundary versus the albumin concentration yielded 0.171 per 1 g % change in albumin concentration. The γ-globulin concentration was also determined directly after eliminating the δ-boundary by adding water to the dialyzed concentrated cerebrospinal fluid(9). With increasing amounts of water, the $(\gamma + \delta)$-peak decreased until at 14% water, the appearance of small spikes at the rear ends of the patterns indicated convection currents due to the negative salt boundaries (Table I). At this point, the values for γ-globulin fell between 4 and 12% of the total area, the range bracketed by (I) and (II).

The apparent percentages of the components at different concentrations of total protein of the same sample of fluid are shown in Table II. The percentage areas for a_2 and β-globulins are approximately the same for all protein concentrations. At the highest protein concentration (2%), the X-protein is about twice and the γ-globulin about one fourth as large as observed at the lowest concentration (0.5%). The data in Tables II and III are uncorrected for boundary anomalies. It would be difficult to apply more than crude corrections to the apparent concentrations unless the corrections were obtained experimentally from sets of measurements on similarly constituted fluids. From the data shown in Table II, the true values for

X-protein and γ-globulins in Tables III, V and VI may be estimated to be $\frac{1}{2}$ to $\frac{2}{3}$ and 1 to 2 times their respective apparent values.

Analyses in phosphate and veronal buffers are compared in Table III. The corrected values for X-protein in phosphate are slightly greater than those in veronal. The uncertainty in determining the size of the δ-boundary in the phosphate buffer is reflected in the lack of agreement of the γ-globulin values in the two buffers.

The relative migration velocity of X-protein with reference to the velocity of albumin V_X/V_{Alb}, depended on the concentration of albumin (Table IV). This relationship agreed qualitatively with theoretical considerations(7,8). The relative velocities of the other components were as follows: a_2, 0.79; β, 0.57; τ, 0.42; the γ-globulin boundary was poorly resolved from the σ-boundary. The corresponding values for plasma were: a_2, 0.84; β, 0.55; $\emptyset$, 0.30 and γ, 0.24. In one cerebrospinal fluid, the γ-globulin migrated much more slowly than the γ-globulin of

TABLE IV. Relative Migration Velocities of X-Protein and Albumin.

Conc. in cell, g %		V_A*	V_X*	V_X/V_A
Protein	Albumin			
.5	.3	1	1.24	1.24
1.3	.7	1.08	1.25	1.15
2.6	1.4	1.14	1.28	1.12

* Arbitrary units.

TABLE V. "General Type" Cerebrospinal Fluid Patterns.

| Total protein, Original (In cell) | | | % | | | | mg protein/100 ml | | | |
Range	Mean		I Range	I Mean	II Range	II Mean	I Range	I Mean	II Range	II Mean
0-29 (400-1300)	20 (800)	X	7-17	11.7	6-16	11 (36)	1.3- 3.8	2.4	1.2- 3.5	2.2
		A	47-72	58.9	43-69	55.4(36)	4.4-19.5	12.1	4.2-18.7	11.4
		a_2	4-16	8.5	4-15	7.7(33)	.8- 2.8	1.7	.8- 2.6	1.6
		β	6-22	12.1	5-20	11.2(33)	.8- 5.1	2.5	.8- 4.6	2.3
		γ	0-18	8.9	8-27	14.7(36)	0 - 4.4	1.7	1 - 5.4	2.9
30-39 (600-1300)	35 (1100)	X	6-20	11.2	5-13	10.1(13)	2.1- 5.9	3.9	1.9- 5.9	3.5
		A	50-70	61.8	43-67	56.6(13)	16.5-25	21.3	14.2-22.8	19.5
		a_2	5-12	8.5	5-11	7.8(13)	1.8- 4.3	2.9	1.8- 4	2.7
		β	8-21	13.8	8-18	12.8(13)	2.4- 7	4.8	2.4- 6.2	4.4
		γ	0-14	4.7	5-23	12.7(13)	0 - 5.2	1.7	1.6- 9	4.3

TABLE VI. Atypical Cerebrospinal Fluid Patterns. The italicized figures represent deviations from "general type."

| Protein conc., mg % | Apparent concentrations (%) | | | | | | | | | | | | Diagnosis |
	X I	X II	A I	A II	a_2 I	a_2 II	β I	β II	τ I	τ II	γ I	γ II	
54	10	10	69	64	5	4	11	10	5	5	0	7	Encephalitis
400	3	3	53	48	*		6	5	4	4	34	40	Lymphocytic meningitis
19	15	14	40	37	14	13	10	9	6	6	15	21	Epilepsy
26	21	19	56	48	9	7	9	7	3	3	2	16	Convulsions, undiagnosed
29	10	9	53	48	8	8	16	14	**		13	21†	" "
29	15	12	56	47	8	7	3	3	1	1	17	30	Cortical atrophy
29	11	10	53	47	11	10	10	9	3	3	12	21	Jacksonian epilepsy
34	4	3	35	30	17	14	12	10	**		32	43	Headache, undiagnosed
28	7	6	48	43	10	9	8	8	**		27	34	Pia arachnoiditis
30	11	10	52	47	9	8	9	8	**		19	27	Epilepsy
18	6	6	45	39	12	11	10	9	5	4	22	31‡	"
22	7	7	58	56	9	8	9	8	**		17	21§	Non-thrombocytopenic purpura
28	10	9	59	54	10	9‖	9	8	4	3	8	17	Meningitis
28	16	15	66	59	5	5	10	9¶	**		3	12	Encephalopathy
22	10	9	62	58	9	8	19	18¶	**		0	7	Subdural hemorrhage
26	12	11	60	54	8	7	18	17¶	**		2	11	Epilepsy

* Low conc. of a_2, included in β-globulin area. † High γ-globulin, separated from δ-boundary.
‡ 3 γ-globulins observed. § 3 γ-globulins observed, separated from δ-boundary. ‖ a_1- and a_2-globulins with increased mobility. ¶ Additional slow β-globulin. ** τ-component included in β-globulin area.

plasma in both phosphate or veronal.

A component, designated as X_1-protein, which migrated 8 to 14% faster than X-protein, was observed in 9 cerebrospinal fluids. A small elevation or a spike, representing either a low concentration of X_1-protein or a convectional disturbance, was present in 4 samples.

Electrophoretic analyses. The majority of cerebrospinal fluids gave patterns of the type shown in Fig. 1a. The ranges in relative areas and protein concentrations of the components are summarized in Table V; these data were arbitrarily divided into 2 groups according to the protein concentrations of the original samples. The relative concentrations of the components of these 2 groups differed very little. Since the ranges did not differ either, this indicated that no one particular component was responsible for the higher protein concentrations.

In 13 of the 36 fluids in the lower protein concentration group (0-29 mg %) and in 8 of the 13 fluids in the higher group (30-39 mg %), the component between β- and γ-globulin, designated as τ, formed a distinct peak and its area ranged between 3 and 8% of the total area. This component was either missing or present in small amounts in the remaining fluids. In a few cases, a component migrated between albumin and X-protein (Fig. 1b). In one instance, a distinct a_1-component was
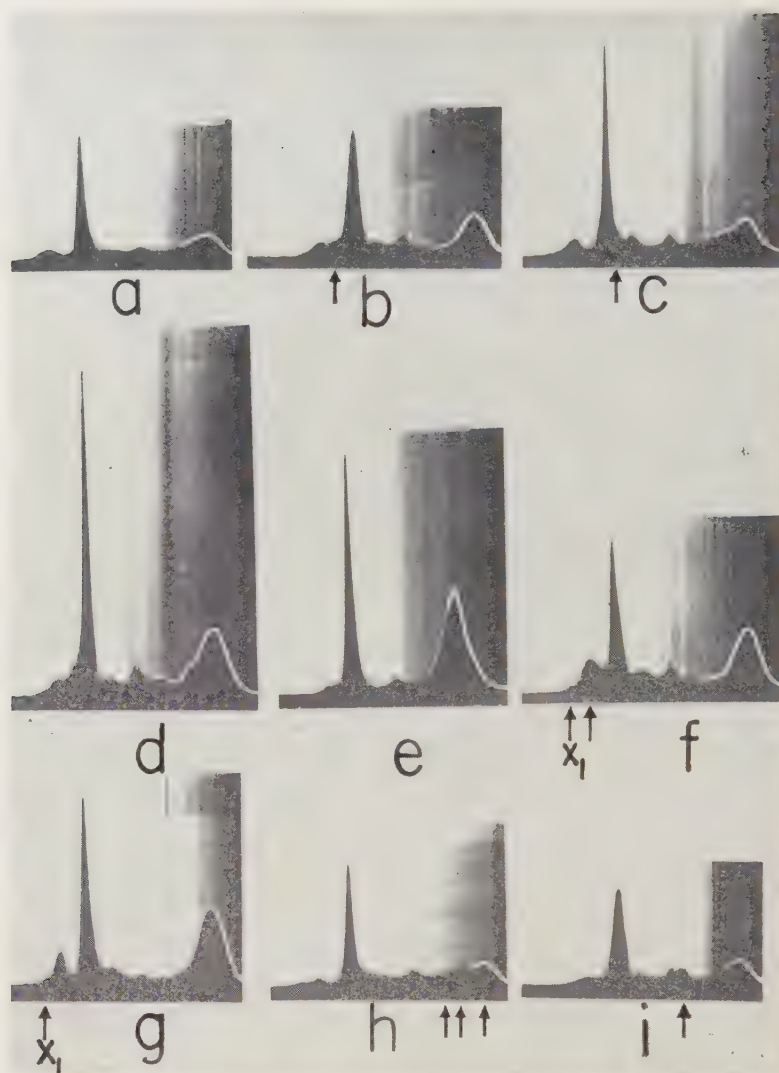
FIG. 1. Atypical electrophoretic patterns of cerebrospinal fluid: (a) Typical pattern; (b) component between albumin and X-protein; (c) α_1 globulin; (d) high total protein; (e) low X-protein, high γ-globulin; (f) high X-protein; (g) low β-globulin, high γ; (h) low X-protein, 3 γ-globulins; (i) additional slow β-globulin.

observed (Fig. 1c).

The incidence of the X_1-component and of the X_1-convection disturbance in the patterns of the cerebrospinal fluids of patients with and without a history of convulsions follows:

	Absent	X_1-protein	Trace of X_1-protein or "X_1-convection"
Convulsive	7	6	3
Non-convulsive	16	3	1

If either X_1-protein or X_1-convections are observed, the patient is more likely to have had convulsions than if X_1-protein is absent ($\chi^2 = 5.0$, P $= 0.02$).

Analytical data for 16 atypical patterns are presented in Table VI. The most frequent changes were: γ-globulin was elevated in 9 fluids, X-protein was decreased in 5 and an additional β-globulin component was present in 3 cases. No relationship could be established between any of these changes and the disease.

Discussion. Bücher *et al.*(5) have demon-

strated the presence of a rapidly migrating component (V) and of a material with a mobility between β- and γ-globulin (τ) in each of 10 cerebrospinal fluids studied with the paper-electrophoresis technic. The rapidly migrating component, designated as X-protein in this laboratory, has been previously described(1) and has been found in each fluid studied in the present investigation. The τ component was noted in less than half of the fluids analyzed. This disagreement with the results of the German workers may possibly be due to the types of patients studied or to the difference in the procedures used for concentrating the cerebrospinal fluid.

Summary. 1. The results of a detailed investigation of the distribution of two rapidly migrating components and of other protein components of cerebrospinal fluids have been presented. A component (X-protein) migrating about 20% faster than the albumin could be demonstrated in every fluid by electrophoresis in phosphate at pH 7.85 and ionic strength 0.05. In addition a component (X_1) migrating 8-14% faster than the X-protein was observed in 20% of the cases. 2. Analyses of 49 patterns of the most frequently encountered type and 16 atypical patterns are presented. No individual protein component was responsible for the higher protein concentration observed in some of the fluids. A possible relationship between the X_1 component and the occurrence of convulsion in patients was indicated.

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Simultaneous Multiplication of Two Different Rickettsiae in the Same Cell. (19967)

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The observations of many investigators have amply shown that infection of a host (bacterial, plant, or animal) by one virus may, under certain conditions, afford temporary protection against infection by another, *i.e.,* interference phenomenon. However, interference is not the universal result of duplicate infections, since many instances of dual virus infections have been described(1,2). Several investigators have reported that two different animal viruses can multiply simultaneously in the same cell. By using viruses that form recognizable intracellular inclusions, studies(3,6) were made to demonstrate cytological evidence that individual cells may be invaded by, and become host to, two different viruses. Levaditi and Schoen(4) observed simultaneous appearance of Negri and Guarnieri bodies in the epithelial cells of rabbit cornea. Syverton and Berry(5) showed that cells of the Shope rabbit papilloma can be superinfected by extraneous viruses. McWhorter and Price(7) demonstrated the presence of inclusions of both tobacco mosaic and tobacco etch viruses in the same cells of doubly infected tobacco plants, and concluded that their presence together indicates simultaneous growth of two plant viruses in the

same cell. Simultaneous multiplication of two bacterial viruses in the same bacterium was also demonstrated(8-10). Pneumonia virus of mice and influenza viruses, or mumps and influenza viruses, have been propagated simultaneously in the respiratory tract of mice and in the allantoic cavity of chick embryos, respectively(11). It has been reported(12) that both influenza A and B viruses have been carried simultaneously through consecutive passages in the chick embryo. Takemori(13) observed in mixed tissue culture that the virus of lymphogranuloma venereum and murine typhus rickettsiae grow simultaneously in the same cell.

The obligatory intracellular parasitic nature of rickettsiae makes the demonstration of dual rickettsial infections in single cells of some theoretical interest. Since rickettsiae can easily be seen microscopically in stained preparations, dual rickettsial infection of the same cell can readily be recognized. Experiments reported here demonstrate the simultaneous multiplication of both *Rickettsia tsutsugamushi* and *Rickettsia typhi* in the same cell.

Materials and methods. The Wilmington strain of murine typhus (*R. typhi*) and the KMT strain* of scrub typhus (*R. tsutsugamushi*) were employed in the experiments. A 10% suspension in buffered saline of mouse livers and spleens infected with *R. tsutsugamushi* was mixed with equal volume of a 10^{-3} dilution of chick embryo yolk sac infected with *R. typhi* and 0.2 ml amounts of this mixture were injected intraperitoneally into 5 mice. At 7 to 10 days after infection when the signs of disease developed, the mice were sacrificed, a 10% suspension of infected livers and spleens was prepared in buffered saline and passed intraperitoneally to 5 new mice as above. Thus far 5 serial passages have been made. Since the two rickettsiae are known to multiply similarly in the cells lining the peritoneal cavity, smears were prepared from the surface of the spleen or the peritoneum, and stained with Giemsa.

Results. Microscopical examinations of the stained smears from each passage revealed,

* Isolated by Prof. N. Ogata in 1951 in Akita Prefecture.

besides cells containing either *R. tsutsugamushi* or *R. typhi,* cells showing simultaneous multiplication of both *R. tsutsugamushi* and *R. typhi* in the cytoplasm.

Some of such doubly infected cells are shown in Fig. 1 A-F. The respective rickettsiae in the same cells can easily be identified, being different in size, form, staining properties and location. *R. tsutsugamushi* is thicker than *R. typhi*, ellipsoidal, often appearing as a diplococcus, colored deep purplish with Giemsa stain, and usually located near the nucleus. It must be emphasized that apparently the two rickettsiae grow quite independently in the same cytoplasm, each forming respective "colonies." Although the two rickettsiae have been carried simultaneously through 5 consecutive passages in mice, apparently without interfering with the growth of each other, maximal growth of *R. typhi* in some of the doubly infected cells seemed to reduce the normal multiplication of *R. tsutsugamushi* in the same cytoplasm. Takemori (13) observed simultaneous growth of the virus of lymphogranuloma venereum and *R. typhi* in the same cell of mouse embryonic tissue in mixed tissue culture. As the microphotographs were not taken at that time, two such doubly infected cells, in schematic form, are also shown in Fig. 1 G for the sake of comparison.

Discussion. This is the first time that dual infections of individual cells with two different obligatory intracellular parasitic agents have been observed microscopically with absolute certainty. In this connection it is of interest to note here that double and multiple infections of single cells with one virus were often reported in the case of the viruses of the psittacosis-lymphogranuloma group(14) and the possibility must be considered that similar double and multiple infections of cells may occur with either one or two animal viruses of other groups. With the use of the electron microscope(15) attempts were made to analyze the changes produced in cells by various viruses. Particularly interesting is a composite picture of a cell(15) which shows but a portion of the cell completely destroyed and disintegrating with virus lying free. The intact portion of the cell still preserves the nor-
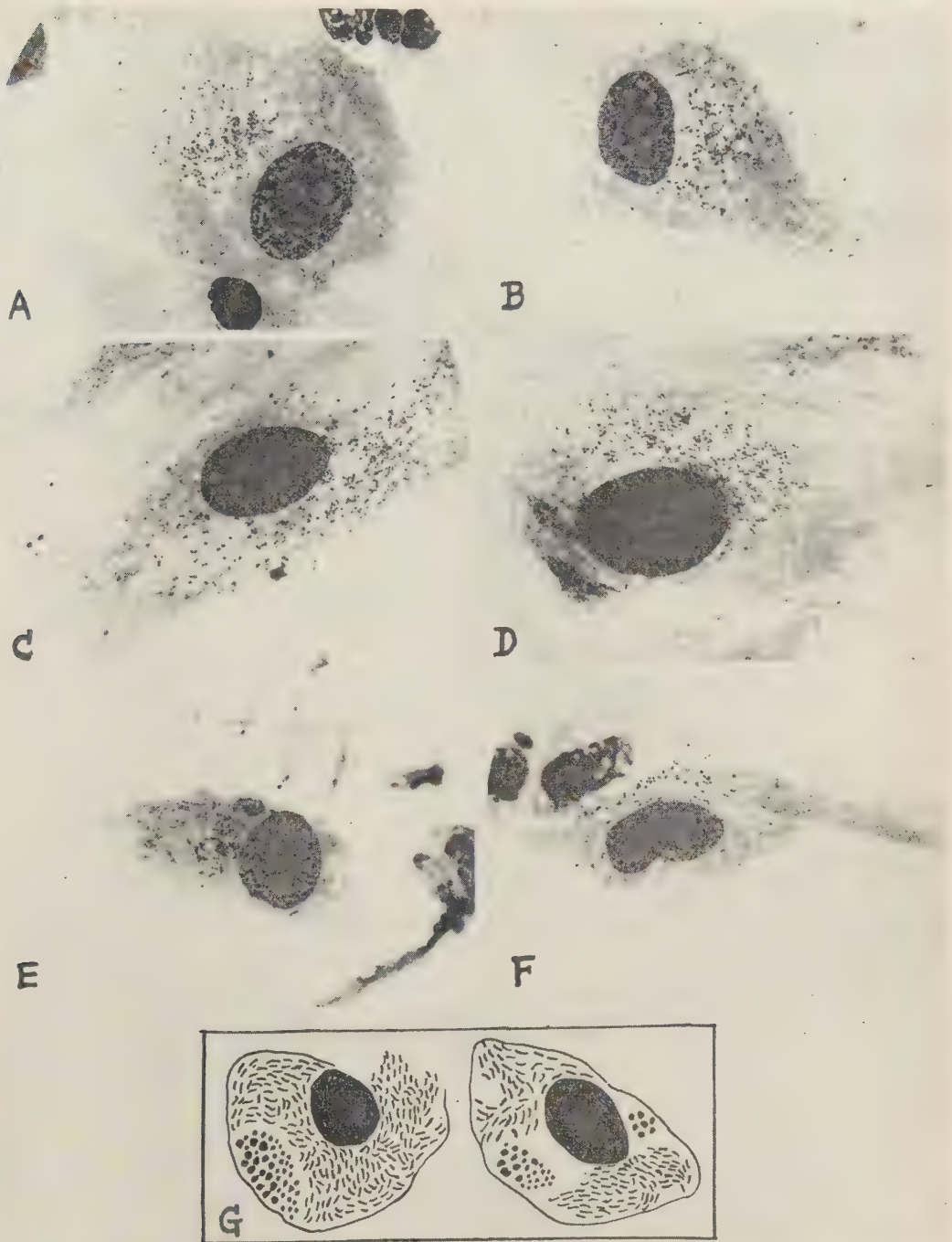
FIG. 1. A-F. Microphotographs by S. Noda. Simultaneous growth of *Rickettsia tsutsugamushi* and *Rickettsia typhi* in the same cell. Smear, stained with Giemsa. G. Simultaneous multiplication of *Rickettsia typhi* and the virus of lymphogranuloma venereum in the same cell (in schematic form). 1000 ×.

mal detailed fibrous microstructure and it seems reasonable to assume that this portion of the cell may become host to a second virus simultaneously inoculated.

The results obtained in these experiments suggest a probable explanation of the mechanism underlying the simultaneous consecutive passages of two animal viruses reported by previous investigators(11,12).

Summary. When mice were inoculated intraperitoneally with a mixture of *R. tsutsugamushi* and *R. typhi* it was found that both rickettsiae multiply simultaneously in the cytoplasm of the same cells lining the peritoneal cavity. The significance of this finding with regard to the simultaneous growth of other viruses in the same cell is described.

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Suppression of Gastric Acidity by Radio Krypton.* (19968)

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Radioactive Krypton, when contained in a balloon inflated in a dog's stomach, provides a safe, convenient procedure for suppressing gastric acidity. The induction of achlorhydria by roentgen therapy has been discarded because of inability to achieve adequate, intense selective radiation; the liver may suffer damage and necrosis, and in a few instances fatality has resulted(1,2). Simon(3) reported the use of P^{32} in suppressing acidity; while effective, the method was technically difficult because it involved the use of a double balloon with the phosphorus absorbed into cotton cemented to the outside of the inner balloon. McKendry(4) and Forse, McKendry and Webster(5) have used I^{131} for the same purpose, instilling it in solution so that it formed a thin film between the walls of a double

walled balloon. In a series of well controlled experiments they were able to decrease the volume of secretion by gastric pouches without significant alteration of pH. *Exact local radiation* can be given only by an isotope with beta and no gamma emission. Most beta emitters such as P^{32} cannot be used inside a balloon because they must be dissolved in water—the water absorbs more than 90% of the radiation from the isotope in balloons of adequate size. However the absorption of beta radiation in a gas-filled balloon is negligible.

Two preliminary survey experiments were done with Argon 41 because of its 1.2 Mev beta emission. The dosage unit used is the "roentgen equivalent physical" or "rep" defined as that amount of beta radiation which, under equilibrium conditions, releases in one gram of air as much energy as one roentgen of gamma rays. A beta dosage of about

12,000 rep to a Heidenhain pouch in one dog reduced the volume of histamine stimulated flow from 35 to 3 cc and changed the pH from 1 to 6 and the total acid from 140 units to 15 units. A Levine stomach tube with a condom tied to the end was placed within the stomach of a second dog. In five weekly treatments a dose in the neighborhood of 20,000 rep to the first 0.5 cm of mucosa was given in order to obtain an idea of what extreme doses would do. Gastric pH rose from 1 to 7. The dog ate most of his ordinary diet until the last two treatments when his daily consumption gradually dwindled until he refused to touch any food. The dog was sacrificed and the stomach examined by our pathologist, Dr. John T. Godwin. "Grossly the rugal folds are lost and the mucosa greyish and granular with minute hemorrhagic foci. Microscopically the mucosa is lost, being replaced by a fibrino-purulent layer. This extends into the submucosa in some areas. Beneath this are extensions of capillaries growing inward from the muscularis mucosa. There is proliferation of fibroblasts with large nuclei which are frequently encountered in post-radiated tissues. The submucosa is greatly thickened due to edema and a few inflammatory cells are scattered about." The use of radio Argon was discontinued because of its intense gamma emission which, while contributing little additional dose to the stomach, constituted a hazard to the operator.

Control experiments in 2 dogs in which balloons inflated with 350 cc of air were left in the stomach for 90 minutes weekly for 6 weeks showed no change in the initial pH of 1 or in the total acid which measured 125 to 140 units.

Krypton 87 has a 78-minute half life, emits beta radiation only, with a maximum energy of 3.2 million electron volts and penetrates tissue to a depth of about 1 cm. Krypton is forced into an aluminum container that is secured in the interior of the Brookhaven nuclear reactor. At the time of treatment several hundred cc of gas are collected in a copper coil; the coil sits in a Dewar vacuum flask and is immersed in liquid nitrogen so that the gas is frozen as it enters and builds up no pressure in the coil which holds 115 cc of gas

at N.T.P. The coil may then be rapidly warmed, creating pressure within which is read on a gauge connected to the system.

Krypton 87, blown into a balloon at the end of a stomach tube, has been used in 2 dogs. Dog 1 required 4 treatments; dog 2, 7 treatments to suppress acidity. In each case the gastric juice was tested after a 24-hour fast and after the injection of 0.2 mg of histamine. From an initial pH of 1 and a total acid of 135 units, the pH has been elevated to 6 and the acid reduced to 12 units. The dogs have remained well, have eaten normally and lost no weight. The reaction of the gastric juice remains unaltered at pH 6 three months after treatment. Upon surgical exploration the gastric mucosa appeared grossly normal, and histologic examination showed no marked changes. "The mucosa of Krypton Dog 1 appears essentially normal with the exception of a mild mononuclear infiltration of the mucosa and scattered foci in which occasional glands have been lost. These glands have been replaced by a loose fibrous tissue. The parietal cells appear in normal numbers."

"Krypton Dog 2 showed some loss of normal architecture in several sections of the stomach wall. Some areas show a loss of glands which are replaced by a loose fibrous tissue containing scattered mononuclear inflammatory cells. In one area the mucosa has been destroyed and replaced by fibrous connective tissue which is covered by a single layer of epithelium. There are adjacent areas of epithelial hyperplasia with atypical glandular formations. The glands do not contain parietal cells and in addition the residual normally formed glands contain fewer parietal cells than those of a normal stomach."

Each treatment consists of 350 cc of Krypton instilled for 78 minutes (one half life). Specific activity is 30 microcuries of Krypton 87 per cc of gas. Krypton 85 is associated with Krypton 87—this is a weak gamma and beta emitter, has a 4.4 hour half life and makes no significant addition to the dose delivered.

Dosage delivered can be only very roughly approximated. Assuming the area of the dog's stomach to be 250 sq cm (Krypton Dog 2 was larger—area about 325 sq cm), and 1

cm penetration of the beta radiation, then 1 liter of Kr-87 will deliver about 300 rep to the gastric mucosa. However, half the total energy is absorbed in the first 0.15 cm of mucosa (half value thickness—0.032 $E_M^{1.33}$); this means about 2,000 rep were absorbed by this superficial initial portion.

Krypton is inert and balloon breakage would cause no serious damage. The length of each treatment and number of treatments required are reasonable. Blood counts in all animals remain normal. The chief difficulty is that at present the work must be confined to the neighborhood of a nuclear reactor.

Further more quantitative animal work and long time observation are required before advocating this treatment in hyperacidity associated with peptic ulcer. At present no contraindications have appeared.

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Effect of Substituted Hydrazines and Related Compounds on Myeloid Mouse Leukemia C-1498.*†   (19969)

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Other investigators(1-5) have extensively studied basic nitrogen compounds comprising free amines, diaryl and dialkyl amines, guanidines and amidines in the chemotherapy of cancer. No comprehensive study has been made with compounds incorporating the hydrazine group. The ease with which hydrazines form hydrazones by conjugation with carbonyl compounds might possibly upset biological systems important for the promotion of tumor growth, particularly oxidation systems such as the citric acid cycle, wherein hydroxy-containing compounds are enzymatically oxidized to the corresponding carbonyl. It is possible that this hydrazone, resulting from the interaction of the hydrazine with the carbonyl group, might interfere with an essential oxidation-reduction process in the cell.

Likewise, hydrazines react readily with glucose; in this way they might also interfere with intracellular carbohydrate metabolism. Sixty-six hydrazine and hydrazide derivatives are reported, including the following parent structures: phenyls, biphenyls, stilbenes, quinolines, naphthalenes, aliphatic as well as a few cyclic hydrazides. The compounds were chosen to give the greatest variations of chemical and physical-chemical properties.

Materials and methods. C-57 black mice were implanted subcutaneously with myeloid leukemia C-1498. Therapy was started the same day. Dosage was approximately one-half the maximum tolerated dose. Seven mice were used to evaluate each compound; in doubtful cases the experiment was repeated. The controls died in 12-14 days. The drugs were usually dissolved or suspended in 4% gum acacia solution; when necessary sorethitan monooleate was added to aid solution. Peanut oil was occasionally used. Drugs administered orally were given by stomach tube.

Results and discussion. The results are summarized in Table I, which is self-explanatory. None of the compounds showed any significant prolongation of life of the animals.

Some of the compounds are of particular interest as they are or resemble active biological agents. No. 58 is the hydrazine analogue of urethane; Nos. 52 and 56 are amino acid analogues; Nos. 29-39 inclusive, represent derivatives of phenylhydrazine. Nos. 42, 45, 50 and 51 are the hydrazides of *p*-aminobenzoic acid, *p*-aminosalicylic acid, isonicotinic acid and nicotinic acid, respectively. Nos. 53 and 59 are urea analogues. 3,5-Dioxo-1,2 diphenyl, 4n-butyl-pyrazolidine (No. 66) (Butazolidin[R]) has recently been introduced in the therapy of arthritis.

These hydrazines were prepared in order to examine a large range of physio-chemical properties. With the exception of Nos. 63-66, inclusive, all of the molecules are planar, and cover a large range of basicity. Although none of these compounds are surface acting agents, varying degrees of solubility in both aqueous and lipid medium are noted. This series therefore represents a gradation of partitian coefficients from high to low values. Aliphatic, aromatic and heterocyclic series with their diverse properties are also represented. A number of position isomers are noted, but the exact geometrical requirements of some important enzyme systems probably could not be met. Unfortunately no clue was obtained from these considerations.

Summary. Sixty-six compounds were tested for activity against myeloid mouse leukemia in C-57 mice. These included hydrazines, hydrazides, hydrazones and related com-

TABLE I

EFFECT OF SUBSTITUTED HYDRAZINE AND RELATED COMPOUNDS ON MYELOID LEUKEMIA – C1498

No	Formula	Dosage mg. per animal	Route	No. of treat. per wk.	Increase of life over contr. in days	No.	Formula	Dosage mg. per animal	Route	No. of treat. per wk.	Increase of life over contr. in days
1	(structure) N<CH₃ / NH₂	1.2	I.P.	3	0	19	(structure) CO·NH·NH₂	0.5	I.P.	5	−1
2	(structure) N<COCH₃ / NH₂	2	I.P.	3	0	20	(structure) CO·NH·NH₂	2	Oral	6	−1
3	(structure) NH·NH₂·HCl	1	I.P.	3	1	21	(structure) CO·NH·NH₂	1.4	Oral	5	−2
4	(structure) NH·NH·CO·CH₃	2	I.P.	3	0	22	(structure) CO·NH·NH₂	0.4	Oral	6	−1
5	(structure) NH·N<CO·CH₃ / CO·CH₃	2	I.P.	3	0	23	(structure) NH·NH₂	1 / 1	I.P. / Oral	3 / 3	−1 / −1
6	(structure) NH·N<CH₃ / CH₃	1.5	I.P.	3	0	24	(structure) NH·NH₂·HCl	1	Oral	3	0
7	H₂N·NH (structure) NH·NH₂	0.5	I.P.	3	0	25	(structure) NH·NH₂	0.66	I.P.	3	0
8	CH₃·CO·NH·NH (structure) NH·NH·CO·CH₃	2	I.P.	3	0	26	(structure) CO·NH·NH₂	3	Oral	4	−2
9	CH₃·NH·NH (structure) NH·NH·CH₃·2HI	0.3	I.P.	3	0	27	(structure) CO·NH·NH₂	0.2	Oral	6	0
10	(structure) N·NH₂·HCl	1	I.P.	3	1	28	(structure) CH=CH·CO·NH·NH₂	2	Oral	5	−1
11	H₂N·NH (structure) O (structure) NH·NH₂ 2HCl	1	I.P.	3	0	29	(structure) NH·NH₂	0.15	Oral	5	1
12	(structure) O (structure) NH·NH₂·HCl	1	I.P.	3	0	30	HO₃S (structure) NH·NH₂	6	Oral	6	1
13	(structure) O (structure) NH·NH·CH₃ HI	1	I.P.	3	0	31	O₂N (structure) NH·NH₂	0.15	Oral	5	0
14	H₂N·NH (structure) SO₂ (structure) NH·NH₂	1	I.P.	3	0	32	(structure) Cl NH·NH₂ Cl	0.7	Oral	5	0
15	(structure) C=C (structure) NH·NH₂	3	I.P.	6	0	33	HO₃S (structure) Cl NH·NH₂ Cl	6	Oral	6	0
16	(structure) NH·NH₂	0.22	I.P.	3	0	34	H₂N·NH (structure) NH·NH₂	8	Oral	6	−1
17	(structure) NH·NH₂	0.5	I.P.	3	0						
18	(structure) NH·NH₂ / CH₃	1	I.P.	5	0						

(Table I continued)

No.	Formula	Dosage mg. per animal	Route	No. of treat. per wk.	Increase of life over contr. in days	No.	Formula	Dosage mg. per animal	Route	No. of treat. per wk.	Increase of life over contr. in days
35	$H_2N \cdot NH$—⬡—$COOH$	0.5 / 2	I.P. / Oral	3 / 3	0 / 0	53	$H_2N \cdot NH \cdot CO \cdot NH \cdot NH_2$	0.4	Oral	5	0
36	⬡—N(CH₃)(NH₂)	0.2	Oral	6	1	54	⬡—CH(NH·NH₂)—CH(NH·NH₂)·COOH	1	Oral	3	0
37	HO_3S—⬡—$NH \cdot NH_2$ (CH₃)	3	I.P.	5	0	55	$NH_2 \cdot NH \cdot CH_2 \cdot CO \cdot OC_2H_5$	0.17	Oral	3	0
38	Br—⬡—$NH \cdot NH_2$	0.7	Oral	3	0	56	$CH_3 \cdot CH$(NH·NH₂)(COOH)	0.5	Oral	3	1
39	NO_2—⬡—$NH \cdot NH_2$ (NO₂)	0.5	I.P.	5	0	57	$HOOC \cdot (CH_2)_4 \cdot CO \cdot NH \cdot NH_2$	6	Oral	6	-1
40	⬡—$NH \cdot NH \cdot CO \cdot CH_3$	0.2	Oral	5	0	58	$C_2H_5O \cdot CO \cdot NH \cdot NH_2$	0.07	Oral	5	1
41	⬡—N(CH₃)(NH·CO·CH₃)	1	I.P.	3	0	59	$H_2N \cdot CO \cdot NH \cdot NH \cdot CO \cdot NH_2$	1	I.P.	5	0
42	H_2N—⬡—$CO \cdot NH \cdot NH_2$	1	Oral	3	-1	60	$C_2H_5O \cdot CO \cdot CH=CH \cdot CO \cdot NH \cdot NH_2$	0.5	I.P.	3	0
43	⬡(NH₂)—$CO \cdot NH \cdot NH_2$	0.3	Oral	5	-2	61	⬡—NH·N=C(COOH)(CH₂·CH₂·COOH)	4	I.P.	3	1
44	dl ⬡—CH(NH·NH₂)(COOH)	0.25	Oral	3	-1	62	⬡N—CO·NH·N=C(COOH)(CH₂·CH₂·COOH)	12	I.P.	6	0
45	NH_2—⬡—$CO \cdot NH \cdot NH_2$ (OH)	2	Oral	6	-1	63	(ring structure)	2	Oral	6	1
46	HO—⬡—$CO \cdot NH \cdot NH_2$	3	Oral	6	-2	64	(ring structure)	1	I.P.	6	1
47	⬡(OH)—$CO \cdot NH \cdot NH_2$	0.5	Oral	6	-2	65	(ring structure)	2	I.P.	5	0
48	H_2N—⬡—$SO_2 NH \cdot NH_2$	1	Oral	6	0	66	(ring structure) C_4H_9	4	Oral	6	-2
49	$CH_3 \cdot CO \cdot NH$—⬡—$SO_2 \cdot NH \cdot NH_2$	3	I.P.	3	0	*	Urethane	8	I.P.	3	2.4
50	N⬡—$CO \cdot NH \cdot NH_2$	0.33	Oral	6	-2	*	Potassium Arsenite	0.1	I.P.	3	0
51	⬡(N)—$CO \cdot NH \cdot NH_2$	0.4	I.P.	6	0						
52	⬡—CH₂·C(NH·NH₂)(COOH)	2	Oral	6	-1						

* Both Urethane and Potassium Arsenite were included for comparison.

The compounds were prepared in this laboratory with the following exceptions:– Nos. 3, 10, 29, 36, 38 were obtained from Distillation Products Corp., No. 37 from General Dyestuff Corp., No. 65 from the Dow Chemical Co., and No. 66 from the Geigy Chemical Co.

pounds. None was found active.

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Sugar Alcohols. XXIX. Sorbitan Polyoxyethylene Fatty Acid Esters and Polyoxyethylene Monostearate and Respiration of Kidney. (19970)

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For many years our studies(1) have been designed to elucidate the fate of the sugar alcohols and their derivatives(2) in the animal body. The widespread use of the de-rivatives of sorbitol as non-ionic emulsifiers in such common components of the human diet as ice cream, bread and other bakery products has focused attention upon the effects of these

TABLE I. Effect of Emulsifying Agents on Oxygen Uptake of Kidney.

Control Q_{O_2}		No. determinations		Q_{O_2}		No. determinations	% change	P value
11.9	S.E. .32	23	Tween 80, 1%	11	S.E. .53	11	—7.6	>.1
14.5	″ .38	8	Tween 20, 1%	16	″ .66	8	+9.4	>.05
14.1	″ .46	8	Tween 60, 1%	12.8	″ .79	8	—9.7	>.05
13.5	″ .44	10	Tween 81, 1%	13.8	″ .38	11	+2	>.1
15.2	″ .80	8	Myrj 52, 1%	15.3	″ .60	8	+ .50	>.9

agents upon various structures of the body. Schweingert, McBride, and Carlson(3) fed two polyoxyethylene monostearate products to weanling hamsters at levels of 5 to 10% and observed that their presence in the diet produced a retarded growth rate and other deleterious effects, such as marked diarrhea and changes in the gastrointestinal tract. On the other hand, extensive feeding experiments (2) in this laboratory using rats, monkeys, dogs and mice failed to reveal these effects. Schulz, Madaus and Soehring(4) studied the effect of certain surface active agents similar to Tweens 20 and 80 on the oxygen uptake of yeast cells and *E. coli*. In general, they observed that low concentrations enhanced gaseous metabolism and higher levels impeded it. They attributed the effect to surface activity and correlated it with the molecular weight of the surface active agents.

Owing to the interest in tissue response to these emulsifiers we decided to study the effect of these agents upon the respiration of kidney tissue.

Substances used. The substances used for this study were:

Tween* 80—
  polyoxyethylene (20) sorbitan monooleate
Tween 20—
  polyoxyethylene ″   ″   ″
Tween 60—
  polyoxyethylene ″   ″   monostearate
Tween 81—
  polyoxyethylene (5)   ″   monooleate
Myrj 52—
  polyoxyethylene (40) stearic acid

Experimental. Male rats weighing between

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* These are trade names employed for these substances as manufactured by the Atlas Powder Co. of Wilmington, Del.

300 and 450 g were killed by a blow on the head. The kidneys were removed and sliced. The oxygen uptake of approximately 100 mg of kidney tissue was determined by the standard Warburg technic. The substrate was a modified Krebs-Ringer's solution with 0.2% glucose. Three cc of Ringer's solution was used for the controls, and 0.3 cc of a 1% solution of the various emulsifying agents was added to 2.7 cc Ringer's solution for the experimental runs.

After an equilibration period of 10 min the emulsifying agents were added to the tissue and readings were taken every 15 min for 1 hr. The Q_{O_2} for 1 hr for both experimental and control runs were calculated and compared. The results are shown in Table I.

None of the changes in Q_{O_2} produced by the various emulsifying agents are considered significant.

Discussion. It has been well established that the ionic surface active agents such as benzalkonium chloride, materially interfere with the cellular metabolism of bacteria. Dubos(5) showed in 1946 that trace quantities of Tween 80 permitted submerged and rapid growth of the tubercular bacillus *in vitro.* On the other hand, the presence of small quantities of Tween 20 (0.005%) depressed the metabolism of the organism. Since the emulsifiers of the Myrj and Tween types are being extensively used in foods, and hence coming in contact with the tissues of the body, it is of interest to note that in concentration of 0.1 of 1% these substances do not interfere with the oxidation of glucose in kidney tissues.

Summary. In kidney slices the addition of Myrj 52, and Tweens 20, 60, 80 and 81, do not significantly interfere with the oxygen uptake of kidney tissue as determined by the

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Virus Growth in Tissue Culture Fibroblasts. II. Coxsackie Virus (Group B) in Cultures of Mouse Fat Tissue.   (19971)

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(Introduced by Icie G. Macy.)
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One of the distinguishing features of the B group of Coxsackie viruses is their ability to produce histopathologic changes in adipose tissues of infant mice(1-3). Although virus has been recovered from these tissues, this does not constitute proof that the virus has specifically infected the cells found in embryonal fat tissues(2,3). Current tissue culture technics offer an opportunity to demonstrate: a) whether a group B Coxsackie virus can be propagated in adipose tissues *in vitro* and b) whether there is a viral susceptibility relationship between the cells of the host tissue and fibroblasts derived from it. In this demonstration we have determined the growth patterns and cytopathogenic effects of a group B type 1 Coxsackie virus in tissue cultures of interscapular fat pads of newborn mice as well as in cultures of fibroblasts derived from this tissue.

Materials and methods. Tissue cultures. A modification of the Porter roller flask method(4) utilizing clotted plasma was employed. The nutrient fluid consisted of 10% chick embryo extract and 30% horse serum incorporated in Hank's balanced salt solution. The initial pH of the nutrient medium was adjusted to 7.4-7.6 with 1.4% (isotonic) $NaHCO_3$. Phenol red in a final concentration of 0.002% was used as indicator. To the medium were added 100 units of penicillin and 100 micrograms of streptomycin per milliliter to control bacterial contamination. Two drops of chicken plasma were placed on cover slips

cut to provide for insertion easily into a Porter flask. With a curved-tip pipette 2 tissue explants were taken up in 2 drops of nutrient fluid and added to the plasma on each cover slip. Following coagulation of the plasma, 2 cover slips, each containing 2 embedded tissue fragments, were each placed on the opposite flat surfaces of a Porter flask. They adhered firmly after the flat inner surfaces of the flasks were wet with nutrient fluid. After adding 1 ml of nutrient medium the flasks were tightly stoppered, placed in a rotating drum ($\frac{1}{5}$ rpm) modified to hold the Porter flasks, and incubated at 36°C. For routine maintenance of the cultures, 3 times each week the nutrient fluid was replaced and lysis of the plasma clot was repaired with fresh plasma. To divide cell colonies the cover slips were removed from the flasks and the fibroblastic outgrowths dissected into portions approximately 1.0 x 1.0 mm for replantation. *Source of cultured cells.* One-half millimeter fragments were prepared from the interscapular fat pads of newborn mice. Four explants and their resulting cellular outgrowths were designated primary culture passages. When colonies reached maximum size they were dissected and portions were transferred to other flasks for the second and third passages. After they had attained their maximum sizes colonies of the primary culture passages contained a central area consisting of tissue carried over from the animal host and a surrounding 2-3 mm band of outgrowth consisting of fibro-

GROUP B COXSACKIE VIRUS IN FIBROBLASTS
DERIVED FROM MOUSE INTERSCAPULAR-FAT PADS
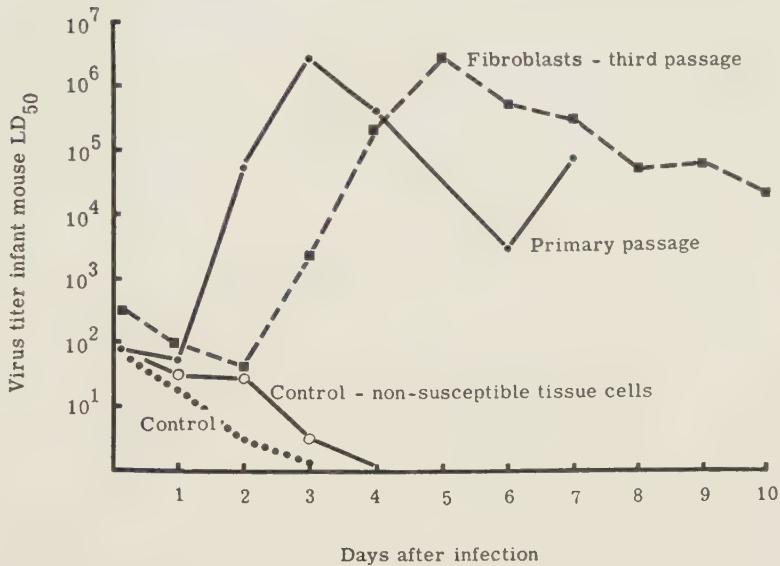
FIG. 1. Growth patterns of group B Coxsackie virus in primary cultures and third passage fibroblasts of mouse interscapular fat pad tissue.

blasts. By the third passage few, if any, of the original cells of the animal host were present and only fibroblasts could be observed microscopically. Although the cultures cannot be said to consist wholly of fibroblasts, we must assume that if other types of cells were present they would be so greatly outnumbered by fibroblasts that the latter must have played the major role in producing the relatively high concentrations of newly propagated virus. For cytological observations, the cover slips containing the colonies were removed from the flasks, washed with balanced salt solution, fixed with Bouin's solution, and stained with Harris' hematoxylin.

Virus. The Connecticut 5 strain of Coxsackie virus, classified as Group B type 1(5), was obtained from the American Type Culture Collection. The virus was received in infected mouse brain in 50% glycerin and was passed once in infant mice before seed preparations were made. For seed preparations, the mice were skinned, eviscerated and the carcasses ground and suspended in 20% dilution in balanced salt solution. Of a 10^{-2} dilution of this suspension, 0.03 ml was inoculated subcutaneously into 2-day-old mice. When

the mice became sick or showed paralysis (4 to 5 days after infection) they were skinned, eviscerated and the carcasses were washed in balanced salt solution, ground and suspended in 20% dilution in balanced salt solution containing 500 units of penicillin and 100 μg of streptomycin per ml. The suspension was centrifuged at 3,000 rpm for 30 minutes and the supernatant fluid was placed in ampules, glass-sealed, and stored in a dry chest until used. *Inoculation of tissue cultures.* The seed preparations of virus were diluted to 10^{-3} in nutrient fluid so that the virus concentration in a 1 ml inoculum was approximately 100 infant mouse LD_{50}. When tissue cultures were 5 to 7 days old the nutrient fluid was removed and flasks containing 4 colonies each were infected with 1 ml of fresh nutrient fluid containing the desired virus concentration, then incubated at 36°C. Culture fluid samples, 0.25 ml, were withdrawn daily and replaced with fresh nutrient fluid to maintain a 1 ml level in the culture flasks. *Titration of viruses.* The samples of virus-infected culture fluids were diluted in tenfold steps with balanced salt solution immediately after removal from the cultures and each dilution was placed
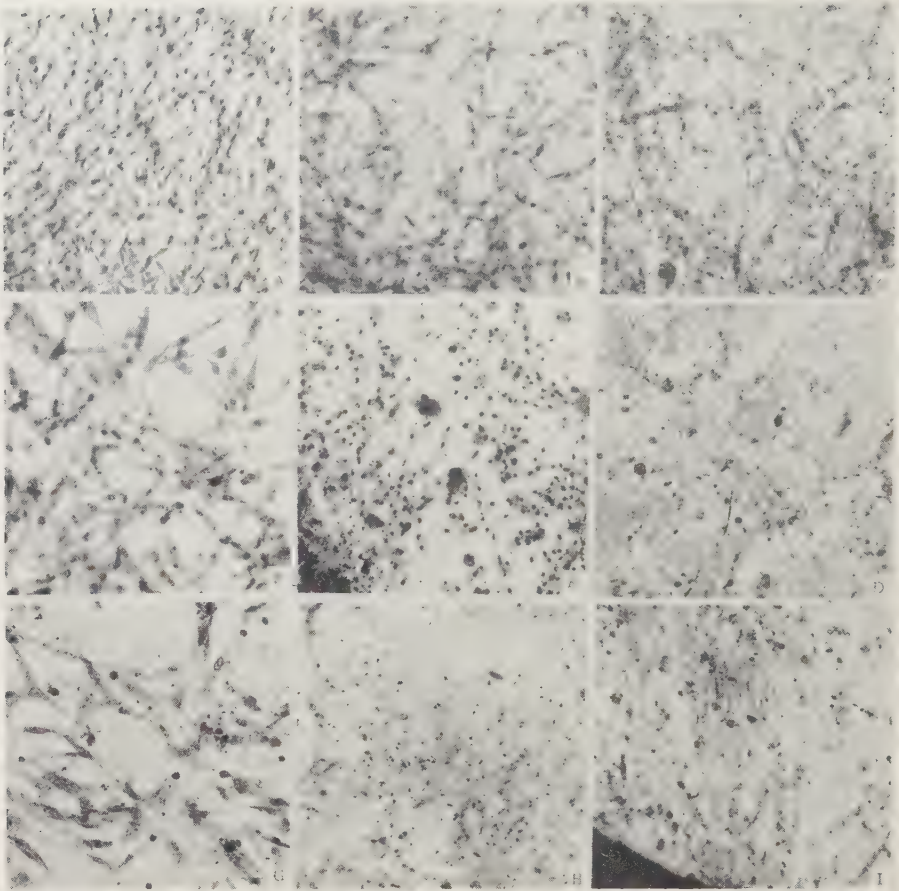
FIG. 2. Normal and virus-infected fibroblasts in cultures of mouse interscapular fat pad tissue. Hematoxylin, $\times$ 150. A. Primary culture, 12 days old, uninfected. B. Primary, infected 3 days. C. Primary, infected 5 days. D. Primary, infected 7 days. E. Primary, infected 9 days. F. Third passage fibroblasts, 9 days old, uninfected. G. Third passage, infected 3 days. H. Third passage, infected 5 days. I. Third passage, infected 7 days.

in an ampule, glass-sealed, and stored in a dry ice chest. This procedure allowed for the inoculation of the various dilutions of virus into 2-day-old mice as litters became available. Of each dilution of virus, 0.03 ml was inoculated subcutaneously into each of 7 2-day-old infant mice. The mice were observed for 2 weeks and 50% lethal endpoints were determined by the method of Reed and Muench (6).

Results. Growth patterns of virus in primary cultures. A series of roller flasks, each containing 4 tissue explants, were incubated for 5 days. By this time the fibroblastic outgrowth had formed colonies approximately 4 mm in diameter. The cultures were inoculated with 1.0 ml of nutrient fluid containing $10^{1.88}$ LD$_{50}$ of virus (10^{-3} dilution of stock virus). Two series of control flasks were inoculated similarly: one contained clotted plasma and nutrient media without cells; the other contained a primary culture of cells from a non-susceptible host, the chick embryo (7).

Fig. 1 presents a typical growth curve of virus in primary cultures of mouse fat pad tissue as demonstrated by infant mouse titrations of tissue culture fluids. After a brief lag the virus titer rose rapidly, reaching a peak of $10^{6.42}$ mouse LD$_{50}$. Thereafter, the titers gradually decreased through the seventh day following inoculation. Viable virus in the control flasks rapidly disappeared, none remaining after 2 days in the flask without cells

or after 4 days in cultures of non-susceptible tissues. The rapid loss of active virus from control flasks, particularly from cell-free controls, has occurred consistently in this laboratory and has been observed with other viruses (8).

Cytopathogenic effects of virus in primary cultures. Although gross evidence of virus infection of the cells was indicated by the failure of the pH to drop as low as that of uninoculated cultures, microscopic observation revealed a direct cytopathogenic effect of virus on the fibroblasts, particularly in stained preparations. The effect was observed first on the third day after infection when the titer of propagated virus was maximal. Fig. 2 shows the progressive cytopathogenesis of the virus on the fibroblastic outgrowth 3, 5, 7 and 9 days after infection and a 12-day-old un-inoculated parallel control culture. Three days after inoculation evidence was present of increased granulation of the cells and a few nuclear remains could be observed after fixation and staining. After 5 days necrosis of the fibroblasts had progressed so far that few healthy cells could be observed in the masses of cellular debris. Seven days after inoculation most of the fibroblasts had been destroyed and the outgrowths were almost completely acellular. By the ninth day another cell type was evident in increased numbers (Fig. 2E). These cells appeared to be healthy and unaffected by virus. At that time a secondary growth of fibroblasts had appeared, but whether these were subsequently affected by virus was not determined.

Growth pattern of virus in third passage fibroblasts. Six-day-old cultures with colony diameters of approximately 4-6 mm were inoculated with $10^{2.5}$ mouse LD_{50} of virus as described for primary cultures. The representative growth curve in Fig. 1 demonstrates that after an initial decrease, the culture fluids began to increase in virus titer on the third day, reaching maximal titers of $10^{6.4}$ mouse LD_{50} 5 days after infection. During the next 9 days the virus titers decreased slightly.

Cytopathogenic effect of virus in third passage fibroblasts. As in primary cultures, evidence of viral destruction of fibroblasts was observed as early as 3 days following

infection (Fig. 2G). In contrast, however, the early cytopathogenic effect occurred 2 days before the maximal titer of virus was observed in the extracellular fluid phase of the culture, but at a time when newly propagated virus could be detected. Progressive destruction of the fibroblasts continued through the fifth and seventh days after infection of the cultures (Fig. 2H, I). Fig. 2F shows the cells of a 9-day-old, uninoculated control culture.

Discussion. The ability of group B type 1, Coxsackie virus to propagate in cultures of adipose tissues of newborn mice suggests that the virus has this ability in the intact host where inflammatory and necrotizing lesions of embryonal fat lobules is a distinctive characteristic(3).

It is evident that in tissue culture the virus was growing in "fibroblasts" derived from the fat tissues. The exact origin of the fibroblasts in these cultures cannot be determined or whether in primary cultures the virus multiplied in the fat cells themselves.

Morphologically, tissue culture fibroblasts are spindle-shaped cells connected with branching cell processes to form a network. Although fibroblasts cultivated from a variety of tissues from a single animal species may be indistinguishable in appearance, the question whether some may be dedifferentiated forms of more mature cells remains unanswered.

The observations with Coxsackie virus parallel, to some extent, those of Scherer and Syverton(9), who showed that poliomyelitis viruses multiply and produce cytopathogenic effects in fibroblasts isolated from monkey testicular tissues. The authors' experiments with influenza A virus(8) indicated that fibroblasts derived from susceptible tissues of the chick embryo do not support the growth of that virus. Herpes simplex virus, which has a broader affinity for different cell types, was grown readily in fibroblasts derived from susceptible tissues of the chick embryo(8). A study of the capacities of different viruses to infect and cause cytologic changes in various cultured tissues and in fibroblasts derived from those tissues might explain some of these problems of virus trophisms and fibroblast origin.

Summary. The ability of a strain of Coxsackie virus, group B type 1, to propagate in cultures of mouse interscapular fat pad tissue and in fibroblasts derived from that tissue was investigated. The virus multiplied in primary cultures containing both explanted host cells and outgrowing fibroblasts, as well as in cultures of fibroblasts carried through 3 tissue culture passages. In each instance the growth of virus was accompanied by progressive cytopathogenic effects which continued until nearly all of the fibroblasts were destroyed.

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Variables in Agglutination and Lysis of Human Red Cells by NDV. (19972)

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An hemolysin associated with the Newcastle disease virus particle has been described by Kilham(1) and also by Burnet and Lind(2). Both groups of workers found that chicken red cells were hemolyzed more readily than human red cells. This paper reports the hemolysis of human O-type red cells by the vaccine strain of NDV and outlines the variables affecting the agglutination and lysis of these cells. Freshly harvested virus did not agglutinate or lyse fresh human red cells but previously frozen virus did both. Furthermore, fresh human red cells which were not agglutinated by fresh virus were agglutinated after storage of the red cells at 4°C for 5-7 days.

Materials and methods. Virus. The Blacksburg or Hitchner(3) strain of NDV which is currently used as a commercial live vaccine for newly hatched chicks, was furnished by Dr. Herald Cox of Lederle Laboratories. This virus had several passages in chick embryos at our laboratory and is referred to here as the Vaccine Strain(4). In contrast to other strains, the vaccine virus causes a transient agglutination of chicken red blood cells and the reaction is inhibited by cold(4). Pools of virus were prepared by allantoic inoculation of 11-day-old embryos and the allantoic fluid was harvested after 2 days of incubation at 35°C. *Human O-type red blood cells.* In most of the experiments, the red cells were from one individual. Blood was obtained by intravenous puncture and put into a large volume of chilled 0.85% NaCl buffered at pH 7.2 with 1% 1/15 M phosphate, without any citrate or anticoagulant. The red cells were washed 3 times with buffered saline and a 1% cell suspension was used in the experiments. *Glassware.* Since the persistence of slight amounts of detergents may give irregular results in the hemagglutination tests(5), no lysol or detergents were used in our laboratory for sterilizing or cleansing glassware. All glassware was cleansed by overnight immersion in chromic acid cleaning solution. Following that, the pipettes and tubes were washed 7 times in running tap water and twice

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TABLE I. Effect of the Vaccine Strain NDV on Fresh Human O-Type Red Cells.

Virus dilution			1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280
Untreated virus	{	H.A.*	0	0	0	0	0	0	0	0
	{	H.O.*	0	0	0	0	0	0	0	0
Virus frozen and thawed	{	H.A.	—	—	—	0	0	0	0	0
	{	H.O.	4†	4	3	1	1	0	0	0

H.A. = Hemagglutination; H.O. = Hemolysis.
† Figure denotes degree of hemolysis on hemagglutination. 4 is max; 0 is neg.

TABLE II. Effect of Calcium Ion on Hemolysis and Hemagglutination.

Virus dilution			1:10	1:20	1:40	1:80	1:160	1:320	1:640
PO₄ buffered saline	{	H.A.	—	—	—	±	0	0	0
	{	H.O.	4	4	3	2	0	0	0
Ca ,, ,,	{	H.A.	2	3	4	4	3	2	0
	{	H.O.	0	0	0	0	0	0	0

in distilled water. The glassware was then dried and used. *Titrations.* All titrations were carried out in 0.85% NaCl buffered at pH 7.2 with 1% M/15 phosphate. Serial 2-fold dilutions starting with a 1/10 dilution were set up in 10 × 100 mm Wassermann tubes. Agglutination or hemolysis was graded as 0 to 4+ by direct examination. The endpoint of the titration was considered to be that dilution which produced at least a 2+ reading.

Results. Effect of the virus on fresh human red cells. Freshly harvested virus or virus left at 4°C for 2 weeks did not agglutinate or hemolyze fresh human red cells. However, the same batch of virus after it had been frozen at −70°C in a CO₂ box did hemolyze the same red cells (Table I).† Hemolysis occurred at room temperature within 30 minutes after the virus and red cells were mixed. The treated virus (previously frozen) lysed the red cells so completely that it was impossible to determine whether any agglutination took place. Hemolysis was inhibited by calcium ion and in the presence of calcium the hemagglutination pattern appeared (Table II).

Effect of the virus on "aged" human red cells. Although fresh human cells were not agglutinated by fresh virus, they were ag-

glutinated after being stored at 4°C for 5-7 days. However, the fresh virus did not hemolyse these "aged" red cells. Treated virus agglutinated and hemolyzed the "aged" red cells just as well as the fresh ones (Table III).

Inhibition of hemolysis by specific immune sera. The specific relation of the hemolysis to the virus was tested determining the degree of inhibition by convalescent immune chicken sera. Hemolysis was completely inhibited by immune sera obtained from convalescent chickens inoculated with NDV, either the B strain or vaccine strain. Normal chicken serum or serum from a chicken immunized to swine influenza or mumps had no inhibitory effect (Table IV). All tested sera were inactivated by heating at 56°C for 30 minutes.

Discussion. It seems likely that the hemolysis reported here actually is a property of the Newcastle infection since it was inhibited by specific sera and was a constant property of the virus. The remarkable conversion of a preparation which did not hemolyze cells to one which did merely by freezing and thawing may indicate that this procedure has broken up the virus particles. Indeed it has been previously demonstrated(6) that freezing and thawing of allantoic fluid containing NDV will cause a much larger portion of the virus to remain in the supernatant fluid after high speed centrifugation. However, the present experiments are not extensive enough to rule out other explanations.

† Drs. A. Granoff and W. Henle have recently informed us that a similar effect of freezing and thawing has been observed in their laboratories.

TABLE III. Effect of Vaccine NDV on Different Ages of Human Red Cells.

	Virus dilution			1:10	1:20	1:40	1:80	1:160	1:320	1:640
Virus at 4°C for 7 days	2 day 0–cells	{	H.A.	0	0	0	0	0	0	0
		{	H.O.	0	0	0	0	0	0	0
	7 ″	{	H.A.	0	1	1	3	2	0	0
		{	H.O.	0	0	0	0	0	0	0
Virus at −70°C for 7 days	2 ″	{	H.A.	—	—	—	—	2	0	0
		{	H.O.	4	4	3	1	1	0	0
	7 ″	{	H.A.	—	—	—	2	2	0	0
		{	H.O.	4	4	3	1	0	0	0

TABLE IV. Inhibition of Hemolysis by Immune Sera.*

Serum dilution	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280
Normal chicken	2	2	2	2	2	2	2	2
Ch. homologous imm. ser.	0	0	0	0	0	±	2	2
Ch. heterologous imm. ser. (B strain NDV)	0	0	0	0	0	0	0	0
Ch. swine flu imm. ser.	2	2	2	2	2	2	2	2
Ch. mumps imm. ser.	2	2	2	2	2	2	2	2

* Performed against 8 hemolytic units.

The capacity of the vaccine strain of NDV to agglutinate aged human red cells is clear. However, the titers indicate that this system needs as great a concentration of virus particles to agglutinate these relatively resistant cells as it does to lyse them. Thus it was possible to set up titrations in which lysis of the cells was the only apparent reaction.

This differs from the effect of washed untreated virus on chicken red cells(7), in which agglutination is produced by a fraction of the number of particles necessary to produce hemolysis.

Summary. The vaccine (Blacksburg) strain of NDV harvested from allantoic fluid of chick embryos, after freezing at −70°C and subsequent thawing, agglutinated and hemolyzed human O-type red blood cells. The same virus, untreated, did not agglutinate or lyse fresh human red cells. However, if the red cells were stored at 4°C for 5-7 days, they were agglutinated but not hemolyzed by the untreated virus.

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The Effect of Tubal Ligation on Ovarian Function in the Rat.* (19973)

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In the adult rat the periovarial sac is a double layer of mesothelium completely surrounding the ovary and containing within its cavity the opening of the oviduct. Below the ostium the sac is open for a short distance. Between the first and third days after birth the oviduct moves caudally and begins to coil, thus bringing the edges of the opening closer together. The peritoneal sac is found to be closed as early as the seventh day after birth although the exact time is variable. This study of Kellogg(1) confirms the statement of earlier workers(2-5). Alden(6) on the other hand, maintains that the ovarian sac in the rat has a peritoneal opening near the orifice of the oviduct. In order to prove his contention this investigator closed the opening with fine sutures. He found that 72 hours later the ovarian capsule was greatly distended with fluid. In addition the uterus was distended and vaginal smear demonstrated a proestrous state. By the end of 108 hours the distension had increased further and ovulation had occurred. Examination of the ovaries demonstrated fresh corpora lutea. Gumbrecht and Loeser(7) believe that the estrogenic hormone produced by the ovary reaches its target organ, the uterus, by way of the oviduct. They contend that this route carries the estrogen to the endometrium where it is absorbed. According to these investigators changes can be observed in the thyroid gland two weeks after hysterectomy, tubal ligation, or decapsulation of the ovaries in rats. They describe the histological picture of the thyroid as identical to that found in castrate animals.

In view of the interesting findings reported by earlier workers(1,4-6) we became interested in the possible role of the ovarian capsule in the maintenance of the normal estrous pattern of female rats.

Material and methods. A total of 20 young

female rats weighing between 150 and 180 g, of Sprague Dawley strain, bred and maintained in our laboratory, were used. Daily vaginal smears were followed in each experimental animal for a period of 2 to 3 weeks to establish normal estrous rhythm in each animal. Any rat which showed aberrations of the normal estrous cycle was discarded. Following this control period the animals were operated on under ether anesthesia and both oviducts were ligated with a single cotton suture at the junction of the uterine horn and the fallopian tube. The daily vaginal smears were continued.

In all experimental animals following tubal ligation the pattern of the estrous cycle changed markedly. Seven to 10 days after surgery all animals went into prolonged periods of estrus which were only occasionally interrupted for periods of from 1 to 3 days of proestrous after which they returned to the extremely heavy estrous smear. This pattern

FIG. 1. Normal (left) and tubal ligated (right) rat ovaries and uteri. The capsule has been removed from the right ovary of the ligated specimen.

FIG. 1-a. Overdistended ovarian sac. Hyperemia of ovary, tube, and uterine horn.

was followed in some animals for as long as 3 months.

When the animals were sacrificed the ovarian capsules were found to be grossly distended (1.5 to 2 cm in diameter). The fallopian tubes were somewhat distended and the uterine horns demonstrated estrogenic effect. There was marked hyperemia of the ovary, oviduct and uterus. (Fig. 1, Fig. 1-a)

The fluid from the ovarian capsule was collected and injected into castrated female rats. These animals showed estrogenic activity of the fluid as demonstrated by estrous smears.

When the experimental animals (with tubal ligation) were castrated the vaginal smear reverted to typical diestrous picture on the second or third day postoperatively.

In a second group of rats after the typical pattern of estrus following tubal ligation was well established the animals were again anesthetized and the distended capsule carefully stripped from the ovary. In every case following this procedure the animal gradually returned to the typical estrous cycles of the control animals in 4 to 7 days.

The histological examination of the ovaries, uteri, thyroids, and pituitaries of the experimental animals was extremely interesting. Ovaries were examined after 30 and 90 days following tubal ligation. In the first group (30 days after tubal ligation) the ovaries appear markedly active. Graafian follicles in various stages of development are seen. The cells of the cortex stain deeply. Many mitotic figures are present in the graafian follicles. No new corpora lutea are evident. The medullary portion of the ovary appears unchanged. (Fig. 2)

On the other hand 90 days after tubal ligation marked evidences of degeneration are seen in the ovaries. The cortex contains only a few graafian follicles and the remnants of
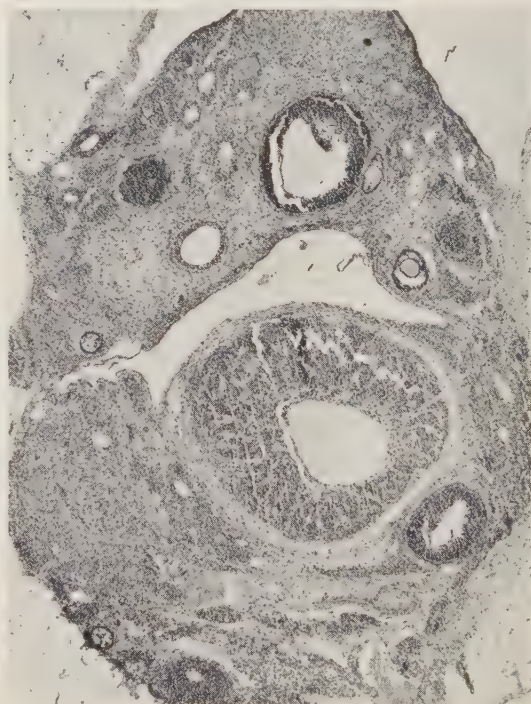
FIG. 2. Graafian follicles in various stages. Many mitotic figures in the follicles. No new corpus luteum.

FIG. 3. Large cystic spaces in the cortex and medulla. Increased connective tissue. Remnants of old corpora lutea.

old corpora lutea. The cells are vacuolated and decreased in size. The amount of connective tissue has increased and dominates the field. Large cystic spaces are present in the cortex and medulla (Fig. 3).

In those cases where the ovarian capsule was removed 60 days following tubal ligation the ovaries demonstrated typically normal histology.

The uterus shows histological changes as well. The muscle fibers, the connective tissue elements, and the endometrium are edematous. The glands are simple tubes without tortuosity, characteristic of estrogenic stimulation (Fig. 4). The vascular structures are distended. In the animals where tubal ligation was permitted to remain for 90 days the histology of the uterus was similar to that of castrate animals in spite of continuous estrogenic response of the vaginal mucosa.

The thyroids appeared enlarged but we were unable to observe the castration changes described by Gumbrecht and Loeser.

Discussion. In view of our findings it is difficult to understand the observation of Alden(6) who states that the ovarian sac has a peritoneal opening near the orifice of the oviduct. One would expect that distension of the sac would not occur by simple ligation of the oviduct at the junction with the uterine cornu. This in no way interferes with the capsule of the ovary.

It is also difficult to understand the findings of Gumbrecht and Loeser(7) who state that the "capsule serves to force the follicular fluid, liberated at ovulation to pass into the uterus where the estrogen will act upon the uterus." They state further that the follicle hormone avoiding the circulation is able, and only in this manner, to act as estrogenic hormone.

While it is altogether feasible to suppose that some estrogen produced by the ovary reaches the uterus in this manner, ligation of the oviduct with resultant capsular distension and uterine stimulation proves that this is not the only route of estrogens from the ovary to the uterus. If one is to postulate a function of the capsular fluid it is logical to presume that it may serve as an aid in the transportation of the liberated ovum to the uterus.

FIG. 4. Edematous uterine wall. Estrogenic response in the glands of endometrium.

The observation of prolonged estrus in animals in which the fallopian tubes were ligated is especially interesting. The manner in which this is brought about is under study at the present time. Whether the apparent increase in estrogen production is the result of increased FSH from the anterior pituitary is to be determined. The lack of corpora lutea formation is also unusual.

The cystic changes that appear in the ovaries of animals 90 days after tubal ligation can probably be best explained on the basis of an inadequate blood supply. As the fluid accumulates in the ovarian sac increased pressure induces ischemia of the tissues with resultant degenerative changes.

Summary and conclusions. 1. Young healthy female rats with normal estrous cycles when treated by simple bilateral ligation of the oviducts proximal to the uterus show prolonged periods of estrus. 2. When the distended capsule produced by this procedure is removed the animals resume normal estrous cycles. 3. The fluid obtained from the capsule contains estrogenic hormone as demonstrated by estrous smears in spayed female rats. 4. The noteworthy histological changes in the ovaries after 30 days of tubal ligation are marked follicle stimulation and the absence of new corpora lutea. After 90 days following ligation the ovaries become cystic, probably due to an inadequacy of the blood supply.

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Inhibition of the Hooker-Forbes Response by Aminopterin.* (19974)

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Numerous papers have appeared in the literature concerning the effects of folic acid antagonists on estrogen and androgen-induced growth in the reproductive tracts of laboratory animals(1-14). Until recently, however, very little experimental work has been directed toward ascertaining the influence of folic acid antagonists (F.A.A.) on the action of progesterone. It was mentioned in a preliminary report from this laboratory(15) that one of the F.A.A. (aminopterin;† 4-amino pteroylglutamic acid) would inhibit the effects of progesterone on the stromal connective tissue cells in the uteri of ovariectomized mice. This paper presents a more detailed study of the effects of aminopterin on the Hooker-Forbes reaction(16), a progestational response elicited by small amounts of progesterone in ligated segments of the uteri of ovariectomized mice.

Materials and methods. A total of 53 Swiss albino mice, weighing 20 to 23 g, were used for these experiments. The mice were maintained in a warm room, and fed the standard Rockland mouse diet. They were ovariectomized via the dorsolumbar route, and 16 days after the operation were given a single intrauterine injection. The folic acid antagonist (aminopterin), estradiol 17-beta and progesterone were dissolved alone or in com-

TABLE I. Effect of Progesterone, Aminopterin, and a Combination of Both on the Hooker and Forbes Assay for Progesterone in the Uterus of the Mouse.*

—Treatment—		Results
μg	μg	
9 P†‡		+
20 A		—
40 A		—
50 A		—
10 A + 9 P§		+
20 A + 9 P		+
30 A + 9 P		+
40 A + 9 P		—
50 A + 9 P		—

* Three to 5 mice per experimental group.
† Total amount inj. .0006 cc.
‡ P = Progesterone. A = Aminopterin.
§ Aminopterin and progesterone combined in amounts indicated and dissolved in 1 cc of physiological saline. Total amount inj., .0006 cc.

binations in an aqueous medium. Forty-eight hours after intra-uterine injection, the mice were killed and the uteri were carefully dissected out, fixed in Lavdowsky's fluid, embedded in paraffin, sectioned at 7 microns, and stained lightly with hematoxylin and eosin. The slides were then examined, and evaluated in terms of a positive (+) or negative (—) Hooker-Forbes response.

Results and discussion. A positive Hooker-Forbes response invariably results when 0.0006 cc of a solution containing 9 μg progesterone per cc is injected into an isolated segment of the uterus of a castrated mouse. However, when the same dosage (0.0006 cc) of a similar solution of progesterone (9 μg/cc) is given to which 40 to 50 μg aminopterin per cc has been added, the action of progesterone is completely inhibited. In similar experiments in which 10 to 30 μg aminopterin per cc was combined with the stock solution of progesterone, there was no apparent interference with the Hooker-Forbes reaction (Table I). In those mice in which the reaction was inhibited, the stromal nuclei had the same appearance usually found in ovariectomized, non-treated mice. Such nuclei were irregularly shaped, dense, and pyknotic in contrast to the oval shaped nuclei containing fine chromatin granules and distinct nucleoli in the non-inhibited progesterone-treated mice. Inhibitory doses of aminopterin when injected alone did not repair the castration atrophy in the stromal nuclei.

These experiments show that aminopterin is quite effective in inhibiting the action of progesterone on the uterine stromal nuclei. Likewise, in other experiments(14), it has been demonstrated that 4-amino PGA inhibits the action of estrogen on the uterus with equal facility. However, it seemed possible that aminopterin in the presence of estrogen and progesterone might show a preference for one or the other steroid in the inhibitory action. Previously, workers in this laboratory(17) reported that estrogen and progesterone in a ratio of 1:200 gave a negative Hooker-Forbes response. This experiment was repeated and confirmed. In addition to the 1:200 ratio of estrogen and progesterone (*i.e.*, 0.0006 cc of a solution containing 0.025 μg estradiol 17-b and 5 μg progesterone per cc), 30 to 50 μg of aminopterin per cc were administered concurrently. Both the response of the stromal nuclei of the endometrium to progesterone and the increase in height of the epithelium of the uterine lumen and glands, characteristic of estrogenic action, were completely inhibited. These results suggest the possibility of a common requirement for the action of both of these steroids (Table II).

The clear-cut inhibition of the Hooker-Forbes reaction obtained by the administration of 0.0006 cc of a solution containing 40-50 μg aminopterin plus 9 μg progesterone per cc strongly suggests that aminopterin is a potent inhibitor of progesterone as well as estrogen. It seems quite probable that the inhibition produced by aminopterin has a direct effect on the action of estrogen and progesterone, and is not due to the toxicity of

TABLE II. Effect of Combinations of Estradiol, Progesterone, and Aminopterin on the Hooker-Forbes Reaction.*

Treatment	Results
5 μg progesterone†	+
Estradiol:progesterone (1:200)	—
E:P (1:200) + 30 μg A‡	—
" + 40 A	—
" + 50 A	—

* Three to 5 mice per experimental group.
† All substances for a particular treatment were combined in 1 cc of physiological saline, and the amount inj. was .0006 cc.
‡ A = Aminopterin.

the inhibiting compound. This is in agreement with the observations of Velardo and Hisaw(18) on the inhibition of decidual development by 4-amino PGA in rats. The present state of our knowledge of the action of aminopterin indicates that it interferes in some way with the enzyme systems associated with the physiological activity of estrogen and progesterone(19-25).

Summary. The response of the uteri of ovariectomized mice to a total amount of 0.0054 μg progesterone was inhibited by the simultaneous administration of 0.0240 to 0.030 μg 4-amino pteroylglutamic acid. Aminopterin (4-amino PGA) showed no selective inhibition for estrogen or progesterone when combined with these two steroids, but inhibited both of these compounds when injected within a ligated segment of the uterus of ovariectomized mice according to the method of Hooker and Forbes.

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Rabies Street Virus Strains in the Syrian Hamster and in the Swiss Albino Mouse. (19975)

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Specific inclusion bodies present in the central nervous systems of animals dying from rabies are called "Negri bodies"(1,2). These are most frequently demonstrated within, but are revealed occasionally outside the nerve cells. A definite diagnosis of rabies is made upon demonstration of these bodies in stained smears. These inclusions are eosinophilic bodies, usually spherical, but of other shapes, varying from 1 to 30 μ in size. The inner structure shows basophilic granules. At present the animal most frequently used for the diagnosis of rabies is the mouse. A positive diagnosis is made on the development of symptoms of rabies in the mouse and the presence of Negri bodies in the mouse brain.

However, the Negri bodies are frequently small and few in number, which sometimes makes their detection difficult. In previous studies the authors found the Negri bodies present in the brains of rabies-infected hamsters to be large and quite numerous.

The present study was undertaken to compare the mouse and hamster as test animals for the diagnosis of rabies.

Materials and methods. The strains of rabies virus used in this study were obtained from Dr. A. N. Metcalf of the Pennsylvania Bureau of Animal Industry at Enola, Pennsylvania. These strains are as follows: 191247 (fox brain), 191248 (dog brain), 191268 (fox brain), 191324 (fox brain), 191427 (cow brain), 191492 (fox brain), 191511 (cat brain), 191512 (fox brain), 191761 (cat brain), 191800 (fox brain), 192482 (dog brain), 192486 (dog brain), 192510 (dog brain), and 192743 (dog brain). These specimens had been proven positive for rabies at the Pennsylvania Bureau of Animal Industry, and were preserved in glycerin until initiation of the present study. The specimens were removed from the glycerin, ground in mortars with alundum, and diluted to 20% suspensions with physiological saline. Each suspension was cultured in thioglycollate broth, and all, except for 191247, were found to be contaminated with bacteria. Since mice are relatively resistant to small numbers of bacteria inoculated intracerebrally, no attempt was made at this time to eliminate the bacterial contaminants. Diagnosis of rabies in hamsters and mice was made on the following factors: 1) virus symptoms present in hamsters and mice and 2) demonstration of Negri bodies in the Ammon's horn of the hamster and mouse brain. The presence of Negri bodies was determined by staining touch preparations of the Ammon's horn with Sellers' stain(3) and examining the slides under an optical microscope. Fifty-six healthy Syrian hamsters, age 18 days, were divided into 14 groups of 4 hamsters each. Each group was inoculated intracerebrally with one of the rabies-bearing brain suspensions. Each hamster received 0.03 cc. Fifty-six additional hamsters were divided into 14 groups, and each group was administered one of the above suspensions by rectal instillation. Each hamster received 0.1 cc. For the rectal instillation, the end of an 18-gauge needle was filed off, rough ends smoothed down, and the tip lubricated with vaseline before being inserted. Fifty-six Swiss albino mice, age 3 weeks, were divided into 14 groups of 4 mice each. Each group was inoculated intracerebrally with one of the suspensions. The inoculum for each mouse was 0.03 cc.

Results. All hamsters and mice inoculated intracerebrally with specimen 192743 succumbed to bacterial infection. The specimen was treated with ether in an attempt to eliminate the contaminating organisms. However, on reculture, there was still gross contamination, and hamsters and mice inoculated intracerebrally with the ether-treated suspension succumbed within 24 hours. The hamsters which had received the rabies suspensions by rectal instillation remained normal until the 5th to 10th day post challenge, at which time symptoms of rabies were evidenced.

All 56 hamsters injected intracerebrally with the rabies suspensions showed symptoms of rabies between the 6th and 9th days. The incubation periods and the form of rabies (furious or dumb) for each strain are given in Tables I and II. The animals were sacrificed when symptoms of rabies appeared. By using the Sellers' stain technic numerous Negri bodies were found to be present in the brains

TABLE I. Response of Hamsters Inoculated Intracerebrally with Rabies Street Virus. Four hamsters injected in each series. Numerous Negri bodies in each series.

Virus strain	Min to max incubation period, days	Type of rabies	
		Dumb	Furious
191247	7		Yes
1248	7		ʺ
1268	8–9	Yes	
1324	9	ʺ	
1427	8	ʺ	
1492	8	ʺ	
1511	7		ʺ
1512	6		ʺ
1761	8–9	ʺ	
1800	6		ʺ
2482	8	ʺ	
2486	6	ʺ	
2510	8	ʺ	
2743	C*		

* C = Contaminated.

TABLE II. Response of Hamsters to Rabies Street Virus Administered Rectally. Four hamsters exposed in each series. Negri bodies numerous in each series.

Virus strain	Min to max incubation period, days	Type of rabies	
		Dumb	Furious
191247	10	Yes	
1248	5–7		Yes
1268	8	,,	
1324	10	,,	
1427	9	,,	
1492	9	,,	
1511	10	,,	
1512	10	,,	,,
1761	10	,,	
1800	10	,,	
2482	10	,,	
2486	10	..	
2510	10	,,	
2743	9	,,	

TABLE III. Response of Mice to Rabies Street Virus. Four mice exposed in each series. Few Negri bodies in each series.

Virus strain	Min to max incubation period, days	Type of rabies	
		Dumb	Furious
191247			
1248			
1268			
1324			
1427			
1492			
1511	8	Yes	
1512			
1761			
1800			
2482			
2486			
2510			
2743	C*		

* C = Contaminated.

of the infected animals. The mice injected intracerebrally with the rabies strains, showed symptoms of rabies on the 8th day and were examined, utilizing the same method as mentioned above. The results are given in Table III.

Discussion. As noted in Tables I through III, the incubation periods varied somewhat, depending upon the species of animal inocu-

lated and the route of inoculation. With 6 strains the incubation periods were one to 2 days shorter in the intracerebrally inoculated hamsters than in the mice. However, with 3 strains the incubation periods were one day longer in the hamsters than in the mice. With all but 2 strains, the incubation periods in hamsters of the rectal group were one to 2 days longer than in the mice.

Upon examination of the touch preparations, Negri bodies seen in the hamster brains were more numerous and larger than those in the mouse brains.

The advantage of rectal over intracerebral administration of the virus is demonstrated with strain 192743. In this instance, the specimen was so grossly contaminated that all hamsters and mice inoculated intracerebrally, succumbed to bacterial infection within the first 24 hours, whereas those hamsters challenged rectally were unaffected by the contaminants.

Summary. 1. Hamsters and mice were challenged intracerebrally and hamsters rectally with 14 strains of rabies street virus in order to determine the better species of animal and route of inoculation in diagnostic work for rabies. With 6 of the 14 strains the incubation periods in intracerebrally inoculated hamsters were shorter than in the mice. Although the incubation period was longer in the hamsters challenged rectally, no preliminary treatment to eliminate bacterial contamination was necessary. 2. Examination of stained touch preparations revealed that the Negri bodies in infected hamster brains were larger and more numerous than those in infected mouse brains.

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Diphtheria Antitoxin Formation in the Horse at site of Injection of Toxoid and Adjuvants.   (19976)

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Numerous attempts have been made to demonstrate the formation of antibodies at the site of injection of antigen in the skin or subcutaneous tissue. The literature on this subject has been reviewed by Burnet and Fenner(1) and also by Oakley and his associates(2). Employing alum precipitated diphtheria or tetanus toxoids the latter authors found that after a single *secondary* injection of these antigens into the skin, fat or voluntary muscle of rabbits or guinea pigs, specific antitoxin may be formed at the site of the deposition of the toxoids. However, the local formation of antitoxin was not demonstrable in a group of seven horses given a single secondary intracutaneous injection of alum precipitated diphtheria or tetanus toxoids. Evidence for the formation of antibodies in the rabbit at the site of intracutaneous injection of killed tubercle bacilli in paraffin oil was obtained by Westwater(3). He found antibodies fixing complement in the presence of tubercle bacilli in the extracts of the cutaneous nodules before they appeared in the blood.

It may be justified to report the following observation in part because it does not involve the extraction of tissue.

Horse No. 848 was injected repeatedly with concentrated diphtheria toxoid, combined with Falba, paraffin oil and heat killed and dried tubercle bacilli into the deep subcutaneous tissue(4). Some of the injections were made omitting the mycobacteria and in one instance concentrated broth was substituted for toxoid. The 7th, 8th and 10th injections produced areas of inflammation from which fluid could be withdrawn by hypodermic syringe. The fluid was slightly turbid and contained mononuclear cells, polymorphonuclear leucocytes, lymphocytes and a few red blood cells. The eosinophiles were numerous. There were also fragmented, pyknotic cells. The antitoxin titer of the samples of centrifugalized fluid and that of the blood were compared. The titrations were carried out at intervals of from 20 to 50 units by the intracutaneous technic in the rabbit(5). The material injected and the results of titrations are given in the tables.

The results show the concentration of antitoxin was from two to seven times higher in the fluid from the sites of injections of toxoid plus adjuvants than in the blood, while in the fluid from an inflammatory area injected with concentrated broth and adjuvants the anti-

TABLE I. Material and Schedule of Injections.

Inj. No.	Day after 1st inj.	Toxoid Lf*	Falba, ml	Paraffin oil, ml	Killed tubercle bacilli, mg†
1	0	100	.35	1	1
2	1	100	.35	1	1
3	2	200	.7	2	2
4	5	200	.7	2	0
5	6	400	1.4	4	0
6	9	400	1.4	4	0
7	12	800	2.8	8	8
8	14	1000	5.6	16	0
9	72	2000	5.6	16	3
10C	72	Conc. broth	5.6	16	3

* Toxoid was prepared from cultures of diphtheria bacillus grown on broth. The formalized preparation was concentrated by precipitation with acetone. The precipitate was dissolved in saline solution. The vol inj. was twice the vol of Falba.

† Dry wt.

TABLE II. Diphtheria Antitoxin Units per ml of Fluid from Sites of the Injection and of Serum.

Days after inj. of sites			Units/ml Fluid from sites			
No.7	No.8	No.10c	No.7	No.8	No.10c	Serum
12	10		225	375		60
	16			525		75
	18			425		75
	21			350		65
43			375			75
46			170			75
						425*
		8			175	425
		13			175	450

* 68 days after 1st inj.

toxin concentration was lower than in the blood. This observation supports the conclusion that antibodies are formed at the site of injection of antigen combined with adjuvants and is in harmony with the assumption that the role of these adjuvants is to evoke an accumulation of cells about the antigen which produce antibodies(6).

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Occurrence of Tapered Collagen Fibrils from Human Sources with Observations on Mesenchymal Neoplasms.* (19977)

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Collagen fibrils were first recognized in the electron microscope by Hall, Jakus, and Schmitt(1). Since then attention has been focused on their width and striations but there has been little mention of the occurrence of tapered ends. The ends of collagen fibrils from most sources have been described as square or frayed. Gross(2) has noted the presence of an occasional tapered end in preparations of skin collagen, and Porter(3) has mentioned collagen fibrils which he described as having tapered ends in tissue cultures of fibroblasts. It is the purpose of this note to indicate and discuss the relative frequency of occurrence of tapered collagen fibrils from a number of human sources.

Methods. Pieces of tissue, fresh or fixed in 10% formalin were homogenized in a standard Waring blendor or a micro Waring blendor. A drop of the resulting suspension was placed on a collodion-covered specimen grid, allowed to dry and shadowed with palladium. Preparations were examined in the RCA EMU 50 kV electron microscope.

Results. Adult human tissues. Collagen fibrils with tapered ends were encountered only rarely in preparations of Achilles tendon, dura and skin of adult human beings. Tapered fibrils were found in most samples of human endometrium taken at various intervals during the menstrual cycle.

Embryonic human tissue. Almost every collagen fibril from the skin of human embryos (6 and 10 cm crown-rump measurement) was tapered at one end and a significant number were tapered at both ends (Fig. 1). Tapering was present but less frequent in the specimens of embryonic Achilles tendon.

Benign and malignant neoplasms. Seven human fibrosarcomas were examined and tapered collagen fibrils were present in all. The number of tapered fibrils varied considerably among the fibrosarcomas. They were com-

FIG. 1. Collagen fibrils with tapered ends from the skin of a 6 cm embryo. The irregular **round** bodies at the borders were seen throughout this preparation. A much smaller beaded fibril appears at the bottom. Palladium shadowed. ca. 23550 ×.

FIG. 2. Tapered fibril from a human fibrosarcoma. Note the small beaded fibrils. Palladium shadowed. ca. 8370 ×.

mon in one synovial sarcoma. In 2 of the fibrosarcomas almost every fibril had at least one tapered end and a few had both ends tapered (Fig. 2). Collagen fibrils with tapered ends were present in one of 2 sclerosing hemangiomas and were common in a Wilms' tumor. A mature keloid was devoid of tapered fibrils as was one fibroma. Tapered fibrils did occur in one fibromyoma and in one fibroma.

Discussion. Collagen fibrils with tapered ends occurring in sufficient numbers to be found easily in electron microscope preparations were associated with rapidly growing sources of connective tissue. They were abundant in preparations from the human embryo. They were almost invariably present in the rapidly growing mesenchymal tumors examined and in samples of human endometrium. In preparations of adult human skin, Achilles tendon and dura tapered collagen fibrils were rare to absent and in the more slowly growing human mesenchymal neoplasms their presence was variable. This suggests that an abundance of tapered ends is one of the earmarks of newly formed collagen.

Randall, Fraser, Jackson, Martin, and North(4) observed tapered collagen fibrils in preparations made from the umbilical cord of the rat. From this observation they suggested that growth of the collagen fibril may take place by a process of pyramidal accretion. If one assumes that the true end of the native collagen fibril is tapered in contrast to the square or frayed fractured end, then in sites of apparent active collagen formation one is seeing more true fibril ends than in adult "resting" connective tissue. One explanation for this is that the fibrils from these sites are shorter, thus more true ends are seen. In the human being then, short collagen fibrils with tapered ends are relatively common where it would seem that collagen is being actively formed.

Summary. Preparations of adult human skin, Achilles tendon, dura and endometrium; human embryonic skin and Achilles tendon; and benign and malignant mesenchymal neoplasms were examined in the electron microscope for the relative frequency of occurrence of collagen fibrils with tapered ends. It was found that tapered fibrils occurred with greater frequency where collagen apparently was being actively formed.

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A Method for the Assay of Penicillin in Animal Feeds.   (19978)

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The usage of antibiotics in animal nutrition is rapidly increasing. It is obviously desirable to have an assay method for the determination of antibiotics in feeds at the levels commonly employed. A companion paper describes a method for aureomycin(1). Standard turbidimetric and cup-plate methods can not be used for the determination of antibiotic activity in feeds due to the presence of non-antibiotic materials in the unsupplemented feeds which inhibit the assay organism or modify its response to the antibiotic. The presence of these non-specific materials gives erroneously high antibiotic values for supplemented feeds and an apparent value for feeds to which no antibiotic has been added. The successful assay of antibiotics in feeds has been made possible by the use of a simple technic. The feed extracts containing antibiotics are pipetted on thick paper pads which are placed on a solidified inoculated medium. This is similar to the technic originally described by Vincent and Vincent(2) but it has been considerably refined.

The method has gone through a lengthy process of development. An early form of the method was briefly discussed in abstract(3) and enabled the determination of 4 g of procaine penicillin per ton of feed. The change from thick double layers of medium to a
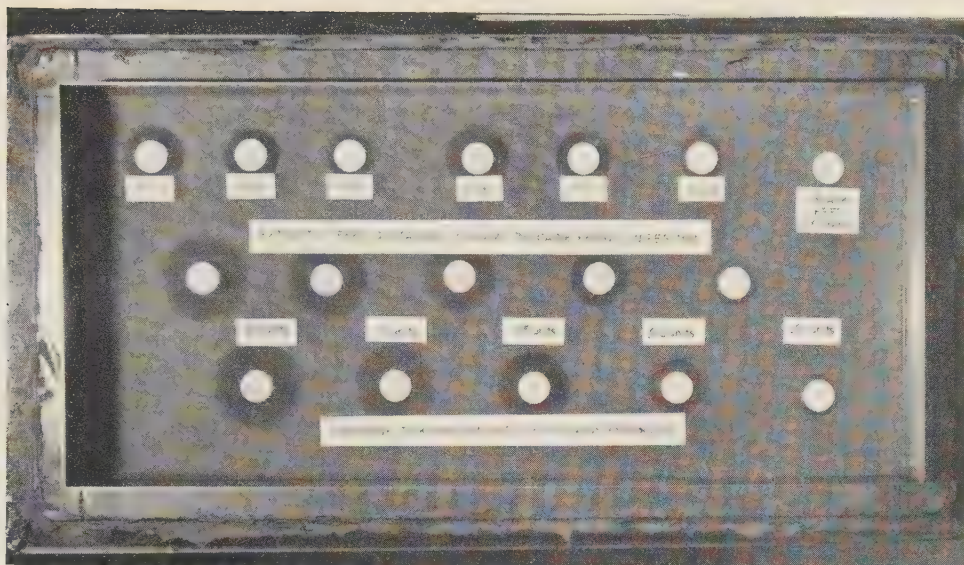
FIG. 1. A typical pad-plate assay after 16 hr incubation at 37°C. The top row of pads contain aliquots of an extract of a feed. The lower 2 rows contain aliquots of the standard extract.

single thin layer increased the sensitivity so that 2 g of procaine penicillin per ton could be accurately determined. The observation by Bigger(4) that sulfonamides increase the sensitivity of staphylococci to penicillin led to the inclusion of 0.15% succinyl sulfathiazole (Sulfasuxidine) in the assay medium which further diminished the minimum amount which can be assayed. The method in its present form is sufficiently sensitive to determine 0.5 g of procaine penicillin per ton while amounts as low as 0.1 g per ton can be detected. The method has been applied to the study of the stability of antibiotics in feed and these results will be reported(5,6).

Methods. Equipment. Flat plates, 14″ x 6″ consisting of stainless steel edges cemented to plate glass bottoms (Fig. 1); a platform with adjusting screws in each corner to provide a level area; filter paper discs No. 740-E, $\frac{1}{2}$″ in diameter (available from Carl Schleicher and Schuell, N. Y.); pointed vernier calipers; and sintered glass funnels of medium porosity (available from A. H. Thomas, Philadelphia. Catalog No. 5593-C.) *Assay organism.* The assay organism is *Staphylococcus aureus* 209-P. It is maintained on agar slants of the same composition as the assay medium (Table II) except that the Sulfasuxidine is omitted and the agar is raised to 1.5%. To

prepare the inoculum for the assay the organism is transferred to inoculum broth (Table II); after 18 to 24 hours incubation at 37°C one ml of the broth culture is used to inoculate 100 ml of assay medium at a temperature of 45°C to 48°C. *Extraction of Samples.* Ten grams of the feed to be assayed are placed in a sintered glass funnel. Twenty-five ml of 99% methanol (1% water) are added to the samples and stirred. The suspension is allowed to filter by gravity into a suction flask for 10 minutes and vacuum is applied. This process is repeated and the combined extracts are diluted with 95% methanol to 50 ml. The extract is further diluted with 95% methanol to give an estimated potency of 0.5 to 1.0 unit of penicillin activity per ml. One-tenth ml of this dilution is pipetted on a filter-paper pad resting on a clean glass plate. This extract is then diluted 1 to 2, 1 to 4, and 1 to 8 with 95% methanol and 0.1 ml of each of the dilutions is pipetted immediately on a filter-paper pad. The solvent is allowed to evaporate and the pads are placed on the assay plate with the aid of a pair of forceps.

Standard. Extracts of feeds which do not contain an antibiotic do not give zones of inhibition; *i.e.*, there is no blank value for an unsupplemented feed. However, if penicillin is added to a methanol feed extract, larger

TABLE I. Comparison of Zones of Inhibition Obtained Using Constant Amount of Penicillin in 3 Variations of the Pad-Plate Method.

Units of penicillin per pad	Double layer, mm	Zone diameter, single layer, mm	Single layer sulfasuxidine, mm
.1	22.2	27.2	33.9
.05	19	23.4	29.7

TABLE II.

Assay medium		Inoculum broth	
Peptone	.6	Peptone	.5
Trypticase	.4	Yeast extr.	.15
Yeast extr.	.3	Beef extr.	.15
Beef extr.	.15	NaCl	.35
Glucose	.1	Glucose	.1
Sulfasuxidine	.15	K_2HPO_4	.37
Agar	1	KH_2PO_4	.13
H_2O	100	H_2O	100

zones are obtained than with the same amount of penicillin added to 95% methanol. This is illustrated in Fig. 2 which will be discussed later. This phenomenon necessitates the use of a standard prepared in either of the following two ways. 1) Fifty mg of procaine penicillin is ground with a few grams of a feed which is of the same general type as the feed being assayed. This thoroughly mixed preparation is mixed with enough of the same feed to give 1 kilo. This is the *Concentrated Standard Feed*. It may be refrigerated and used over a period of 2 months. On the day of assay 10 g of the concentrated standard feed is added to 90 g of a sample of the feed which is of the same composition as the sample to be assayed but which does not contain added antibiotic. This is the *Standard Feed* and if refrigerated, it may be used for 2 months for repeat assays or for assays of the same feed containing different amounts of penicillin. This is extracted in exactly the same manner as the samples. The extract will contain 1 unit of penicillin per ml. One-tenth ml is pipetted on duplicate pads resting on a glass surface. The extract is then diluted 1 to 2, 1 to 4, 1 to 10, and 1 to 20 with 95% methanol and duplicate pads are prepared from each dilution. The solvent is allowed to evaporate before the pads are placed on the assay plate. 2) Ten g of antibiotic-free feed of the same composition as the samples is extracted in the same manner as that of a sample. One ml of 95% methanol containing 0.02 mg of procaine penicillin is added to 19 ml of the feed extract giving a solution containing 1 unit per ml. This is then diluted as described under (1). The above two standards represent a feed containing 4.5 g of procaine penicillin per ton and are satisfactory for the assay of feeds containing from 1.0 to 20 g per ton. If a considerably
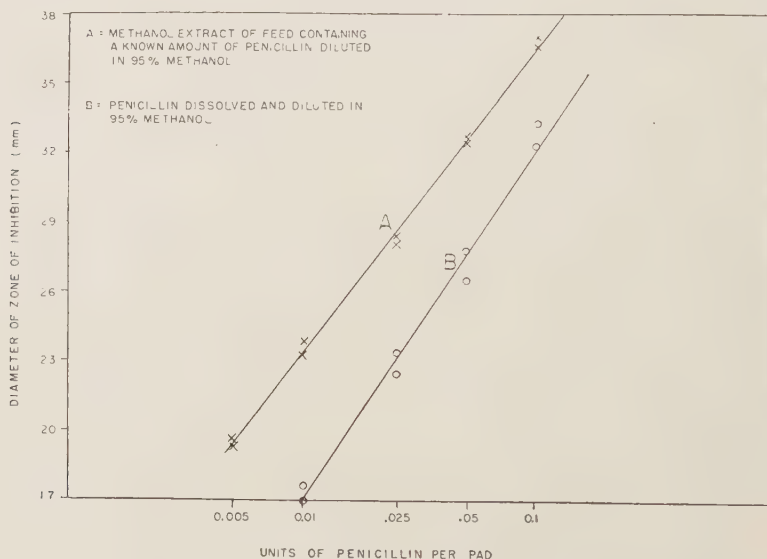
FIG. 2. Comparison of response lines obtained from penicillin contained in a feed extract and in 95% methanol.

TABLE III. Assays of Feeds Containing Known Amounts of Penicillin Salts.

Penicillin salt	Amt added, g/ton	Amt found by assay, g/ton	% recovery
Procaine	23	23	100
	23	21	91
	23	23	100
	10	10	100
	4.6	5	108
	4.6	5	108
	.9	.8	89
NN' dibenzyl ethylene diamine	4.6	4	87
	4.6	4	87
	23	23	100
Di-p-chlorophenylbiguanide	23	22	96
Guanyl urea	23	24	104
		Avg recovery	98%

higher potency feed is to be assayed, adjustments in the potency of the standard should be made so that the amount of feed extract pipetted on the paper pads is within 5-fold of that present on the standard pads. The standard should be prepared from the same type of penicillin salt as is present in the feed or one which approximates it in units per milligram. Procaine penicillin has been used as a standard in assays of penicillin salts which ranged from 895 to 1158 units per milligram with no apparent difference in the response lines when drawn on a unit basis. However, when assaying for a salt which had a potency of 1300 units per mg a definite difference in the slope of the response lines was observed, which may be due to a difference in diffusibility produced by differences in molecular weight. *Assay procedure.* One hundred ml of assay medium (Table II) is prepared and sterilized in a 250 ml Erlenmeyer flask. The medium is cooled to 45°C to 48°C and inoculated with 1 ml of an 18 to 24 hour broth culture of *S. aureus* 209-P. The medium is then poured into a flat bottom plate which is resting on a level surface. After the medium has solidified, the dry filter-paper pads containing the sample and standard dilutions are placed on the agar surface. The assay plate is covered with either a glass plate or a special stainless steel cover made for that purpose and then incubated at 37°C for 15 to 18 hours. After incubation the zones of inhibition are measured with pointed calipers. The standard response line is drawn on semi-log graph paper and the sample values are calculated from it. Line A in Fig. 2 is an example of a typical standard response line. Fig. 1 illustrates a typical assay plate after incubation.

Results and discussion. In order to test the effectiveness of the extraction procedure used in the pad-plate method a number of determinations were made on feeds containing known amounts of various pure penicillin salts. The standard in these experiments was prepared by adding pure penicillin salt to a methanolic feed extract. The results of these tests are given in Table III and show good recovery of known amounts of added penicillin salts.

Repeated tests on refrigerated samples of known content consistently gave an average value within a few percent of the amount of penicillin added. Although earlier reports have shown that penicillin is unstable in the presence of alcohol(7), methanol has been found to be fully adequate as a solvent to extract penicillin from feeds and superior to either water, pyridine, or ether. This apparent anomaly is explained by the data shown in Table IV which shows that two pure penicillin salts added to 1) 95% methanol, 2) an anhydrous methanol extract of undried feed, and 3) an anhydrous methanol extract of *dried* feed were essentially stable. The pure salts added to anhydrous methanol were rapidly destroyed. Water added to the methanol is obviously a stabilizing factor. Since the penicillin salts were stable in an anhydrous methanol extract of feed which had been dried at 100°C for 2 hours, it appears that materials

TABLE IV. Stability of Penicillin in Methanol.

Solvent	Penicillin salt*	% of penicillin remaining	
		After 1 hr	After 4 hr
Anhydrous methanol	Procaine	0	0
	DBED†	0	0
Methanol extr. of undried feed	Procaine	88	93
	DBED	94	92
Methanol extr. of feed dried 2 hr at 100°	Procaine	98	93
	DBED	93	102
Methanol 95% + water 5%	Procaine	102	79
	DBED	92	60

* 5 mg of pure procaine or DBED penicillin was dissolved in 25 ml of solvent and allowed to stand at room temperature.

† Dibenzyl ethylene diamine dipenicillin.

are contained in the feed extracts other than water which protect the penicillin from rapid destruction by anhydrous methanol. The protecting effect of materials in the feed extracts is apparent even after a 25-fold dilution of the extracts with anhydrous methanol. Procaine penicillin retained full activity in such diluted solutions held at room temperature for 4 hours. In a separate experiment procaine penicillin was found to be stable in 99% methanol for 1 hour at room temperature. Formamide also has been found to be satisfactory for extracting procaine and diamine penicillin from feed. This solvent was substituted for methanol in the method and identical assays were obtained on a number of penicillin-containing feeds which had been stored 4 weeks in stability studies.

Fig. 1 shows the assay plate. For demonstration the pads were widely spaced. Routinely 30 pads are put on such a plate permitting the assay of 5 samples; *i.e.*, 4 pads for each sample plus a standard curve in duplicate. The pad showing no zone of inhibition contains 0.1 ml of an extract of blank or unsupplemented feed demonstrating the complete absence of non-antibiotic interference in the pad-plate method from such feeds. Plates larger than the 14″ x 6″ plate shown can be conveniently used since more samples can be assayed with each standard curve.

As mentioned earlier unknown materials in feeds extracted by methanol markedly affect the size of zones of inhibition obtained with various amounts of penicillin, although the feed extracts do not have antibiotic activity in the absence of penicillin. This effect is shown in Fig. 2. To construct line A, 10 g of a poultry feed which contained 4.5 g of penicillin per ton was extracted with methanol, diluted and pipetted as described for samples. Line B was obtained by suitable dilution of a solution of crystalline procaine penicillin in 95% methanol. The mechanism of the effect of the feed extractives on zone size is not known. It appears likely that the extractives either affect the extent of diffusion of the penicillin or enhance its antibiotic action against *S. aureus*.

Crystalline procaine penicillin dissolved in 95% methanol is used for the assay of penicillin feed supplements and a standard response line such as line B (Fig. 2) is constructed. Penicillin is extracted from the feed supplement by suspending 1 g of supplement in 100 ml 95% methanol and allowing contact for 30 minutes at room temperature with occasional mixing. Further dilutions are made with 95% methanol until a 1 unit per ml solution is obtained.

In the aureomycin method the necessity for using a feed for the Standard Feed of exactly the same composition as the samples has been clearly shown (1). With penicillin, however, preliminary results indicate that a single Standard Feed may suffice for the assay of penicillin in a variety of feeds of the same general type; but further investigation is needed.

Some of the fundamental advantages and limitations of the pad-plate method are as follows: Strictly aseptic conditions are unnecessary. Only the assay medium and the inoculum broth are sterilized by autoclaving.

Contamination is rare in plates which have been thoroughly washed. It is not necessary to remove solvents from extracts of samples because they are allowed to evaporate from the pads before the pads are placed on the assay plates. The method is free from interference due to non-antibiotic materials since these non-specific inhibitory materials in the feed extracts do not diffuse into the agar medium but apparently remain on the pads.

Using the single layer technic, it is essential that the plate be resting on a level surface when the agar is poured. Differences in agar depth in different areas of the plate can cause a 2-fold difference in assay values calculated from duplicate pads. In the 2-layer technic, the level surface is not as important since the agar is approximately 6 times as deep and the variations in depth will be small in relation to the total thickness of the agar.

Summary. A rapid simplified technic utilizing inexpensive equipment has been developed for the determination of penicillin in feeds and feed supplements. The method is useful to determine the quantities of penicillin added to feeds for nutritional purposes since it can be used to assay accurately as little as 0.5 g of procaine penicillin per ton of feed. Feeds which are not supplemented with antibiotics do not give blank values. A sample of antibiotic-free feed of the same or similar composition as the samples to be assayed must be available. The test organism is *S. aureus* 209-P which is suspended in thin layers of agar medium upon which paper pads are placed containing methanol extracts of the feeds. The use of the paper pads to contain feed extracts and the use of Sulfasuxidine in the assay medium are the features of the method which permits the method to be used for the determination of the small amounts of penicillin added to feeds.

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Inability of Flavonoids to Modify Roentgen Ray Irradiation Mortality in Guinea Pigs.*† (19979)

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General disagreement exists regarding the usefulness of the flavonoid compounds in the prevention and treatment of Roentgen ray irradiation injury. Rekers and Field(1,2)

reported that rutin modified such damage in dogs but not in rats. However, Griffith *et al.*(3) found rutin beneficial in rats. On the other hand, Kohn, Robinette, and Capp(4) reported that rutin had no effect on Roentgen ray irradiation lethality in rats, and Cronkite *et al.*(5) obtained similar results in mice. Sokoloff *et al.*(6) reported that a citrus flavonoid mixture decreased such lethality in a British brown strain of rats, but Cronkite *et al.*(7) were unable to obtain any benefit in mice. Clark *et al.*(8) reported that "calcium

TABLE I. Effect of Flavonoids on Radiation Mortality in 14 Groups of Guinea Pigs.

Medication and time		Total mortality				
		10 days	15 days	20 days	25 days	30 days
Control		0/20	7/20	7/20	7/20	8/20
PM	Rutin, 250 mg/kg	2/20	6/20	9/20	9/20	9/20
PSM		6/20*	7/20	7/20	7/20	7/20
Control		3/20	10/20	10/20	10/20	10/20
PM	HMC, 100 mg/kg	0/20	7/20	8/20	11/20	11/20
PSM		6/20	13/20	13/20	13/20	13/20
Control		3/20	10/20	11/20	12/20	12/20
PM	Hesperidin, 25 mg/kg	5/20	12/20	14/20	14/20	14/20
PSM		4/20	9/20	11/20	12/20	13/20
Control		0/20	7/20	7/20	7/20	7/20
PM	Naringin, 75 mg/kg	2/20	5/20	6/20	6/20	6/20
PSM		3/20	7/20	7/20	7/20	7/20
PM	MG, 100 mg/kg	3/20	9/20	9/20	10/20	10/20
PSM		3/20	8/20	8/20	9/20	9/20

PM = Premedicated;   PSM = Post-medicated;   HMC = Hesperidin Methyl Chalcone;
MG = Methyl Glucamine.
    * Significant at P = .05.

flavonate" reduced Roentgen ray irradiation mortality in guinea pigs. However, Clark and MacKay(9), upon the basis of their distribution studies of the various flavonoids in the intestinal tract, stated that intestinal decomposition of orally administered flavonoids made it highly unlikely that enough of the drug could be absorbed to produce the effects reported. Dauer and Coon(10) found various flavonoids of no benefit in irradiated rats, mice, and guinea pigs.

We have investigated the protectant action of several flavonoids in Roentgen ray irradiated guinea pigs. These animals were chosen for their high irradiation sensitivity (LD_{50}/30 days, 200 r) and because they are one of the few animal species which seem to require flavonoid compounds as an essential metabolite in their ascorbic acid metabolism(11). The results obtained indicate that the flavonoids used did not modify Roentgen ray irradiation mortality.

Experimental. Female guinea pigs, weighing 286 to 477 g (avg 393 g) were placed upon a high ascorbic acid diet for 4 weeks pre-irradiation and were maintained upon this diet until completion of the experiment. The flavonoids dissolved in saline containing 1% methylglucamine were administered intraperitoneally using alternating sides of the abdomen to reduce the incidence of trauma. The dosages used (rutin, 250 mg/kg; hes-

peridin, 25 mg/kg; hesperidin methyl chalcone, 100 mg/kg; naringin, 75 mg/kg) were based upon those shown to produce the greatest effect upon capillary permeability(12). Both pre- and post-irradiation medication for 10 days was used and the various treatments are shown in Table I. In all cases the control groups received injections of the same volume containing the same amount of methylglucamine as the medicated groups. The 200 r total irradiation dose was administered from above and below the animals with two 250 KVP Picker Industrial units operating simultaneously. The technical factors for both units were: 250 KVP; 15 MA; TSD 40 cm; filters 0.21 mm Cu inherent, 0.5 mm Cu parabolic and 1.0 mm Al; HVL 2.02 mm Cu; size of field—total body; r/minute measured in air 99.4-104.6. The animals were irradiated in groups of 2 or 3 animals each, depending upon the number of animal groups in the particular experiment. Both units were calibrated prior to each experiment with a Victoreen Thimble r meter.

Results. The results obtained with the various treatments are given in Table I. It is quite evident that none of the flavonoid compounds significantly decreased the total radiation mortality. Application of the χ^2 test showed that post-irradiation medication with rutin significantly increased the rate of mortality over that of the controls. The site of

injection, the solubilizer used and the handling of the animals, particularly after irradiation, were not involved in either the rate of mortality or the total mortality because if they had been, definite differences in both of these factors would have shown up in the pre- and post-irradiation methylglucamine groups. The non-irradiated control groups and the saline-methylglucamine and flavonoid injected control groups had no deaths during the experimental period or at any time thereafter up to 60 days. This indicates that the deaths obtained in the medicated irradiated groups were not due to the flavonoids themselves.

Discussion. The results herein reported concerning the ineffectiveness of parenterally administered rutin, hesperidin, hesperidin methyl chalcone, and naringin in modifying Roentgen ray irradiation mortality in guinea pigs confirm the observations of Dauer and Coon(10) who gave rutin, hesperidin methyl chalcone, and citrus vitamin P by the oral route. The results of previous investigators (2,4,7,10) as well as our own would definitely indicate that the flavonoids are incapable of modifying the radiation syndrome in mice, rats, guinea pigs, or dogs.

Summary. It has been shown that rutin, hesperidin, hesperidin methylchalcone, and naringin were ineffective in modifying Roentgen ray irradiation lethality in guinea pigs. There was an indication that post-irradiation medication with rutin may have increased the rate of mortality.

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Species Differences in Circulating 17-Hydroxycorticosteroid Concentrations.* (19980)

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Vogt(1) described a substance in adrenal vein plasma which protected adrenalectomized dogs from cold. Since then, bioassays of adrenal venous plasma have been accomplished by other workers(2,3) and various corticosteroids have been isolated from fresh adrenal tissue(4,5). By the use of recently developed qualitative and quantitative technics, considerable species differences in the steroid content of adrenal venous blood have been demonstrated. By paper-partition chromatography, Bush(6) found that the dog and cat secreted mainly 17-hydroxycorticosterone and some corticosterone; the ferret secreted both compounds in approximately equal amounts; the rat and rabbit produced mainly, if not

TABLE I. 17-Hydroxycorticosteroid Plasma or Serum Concentrations in Various Species.

Species	No. of determinations	Mean, μg %	SE_M	Range
Rabbits	28	0	—	·
Rats	7	2.1	± .96	0 − 7.5
Guinea pigs	10	33.1	± 4.46	18.9–65.6
Humans:				
Children*	31	10	± 1.67	0 −26.8
Adults	13	10.9	± 1.73	3.1–21.6

* Data taken from reference 12.

entirely, corticosterone; the guinea pig mainly 17-hydroxycorticosterone but also corticosterone, 11-dehydrocorticosterone and 11-dehydro-17-hydroxycorticosterone. By chromatographic separation, large amounts of a steroid hormone identified as 17-hydroxycorticosterone have been isolated from the adrenal vein effluent of dogs(7) and smaller amounts from the adrenal venous blood of cows(8). By further refinement of this technic Nelson and Samuels(9) have developed a method for direct quantitation of circulating 17-hydroxycorticosteroids. In normal adult human subjects, they found 17-hydroxycorticosteroid concentrations of 4 to 10 μg per 100 ml of whole blood(10). Using the plasma method (9), which yields values approximately twice those of whole blood, Ely, Kelley, and Raile (11) found a mean value of 10 ± 1.67 μg per 100 ml of plasma in normal children.

In an attempt to find a laboratory animal suitable for 17-hydroxycorticosteroid studies, an investigation of the secretion of these hormones by various species was undertaken. In this report the circulating levels of these compounds in rabbits, rats, guinea pigs, and human subjects are compared.

Materials and methods. Male and female albino rabbits weighing approximately 2 kg, male Sprague-Dawley rats weighing 250 to 350 g, and male guinea pigs weighing 300 to 550 g were used. The human subjects employed were normal young adults. Approximately 30 ml blood samples were obtained from the marginal ear veins of the rabbits and allowed to clot at 4°C. The rats and guinea pigs were rapidly anesthetized in an ethyl ether atmosphere and blood samples were obtained from the abdominal aorta into heparinized syringes. Serum and plasma samples were separated by centrifugation and

stored in deep-freeze until used. Where necessary, the plasma of 2 to 3 rats or guinea pigs were pooled to make 10 to 15 ml samples for the determinations. In addition to the control studies on rabbits, certain response tests were done. One group of rabbits was given a single intramuscular injection of ACTH in a dose of 1.5 mg per pound. Another group was given a single intramuscular injection of 10 mg of cortisone per animal. Blood samples were obtained 2 hours post-injection in the ACTH-treated group, and 2, 8, 24, and 72 hours post-injection in the cortisone-treated group. Circulating 17-hydroxycorticosteroid concentrations were estimated by the plasma method of Nelson and Samuels(9).

Observations. In Table I observations concerning circulating 17-hydroxycorticosteroids in the species studied are summarized. The serum of rabbits was found to contain no detectable amount of these steroids. Rat plasma yielded concentrations of 0 to 7.5 μg per 100 ml with a mean value of 2.1 ± 0.96. Guinea pig plasma contained from 18.9 to 65.6 μg per 100 ml, with a mean of 33.1 ± 4.46 μg per 100 ml of plasma. Plasma of normal adult human subjects had a mean concentration of 10.9 ± 1.73 μg per 100 ml with a range of 3.1 to 21.6 μg.

In Table II, the 17-hydroxycorticosteroid concentrations in rabbits following the injection of ACTH and cortisone are shown. Rabbits given ACTH, 1.5 mg per pound, intramuscularly, were found to have no measurable amounts of 17-hydroxycorticosteroids 2 hours after injection. Following the intramuscular injection of 10 mg of cortisone per animal, 17-hydroxycorticosteroid concentrations as high as 52 μg per 100 ml of plasma were found after 2 hours, with as much as 7.9 μg still detectable at the end of 24 hours. These con-

TABLE II. 17-Hydroxycorticosteroid Serum Levels in Rabbits Following Injection of ACTH and Cortisone.

Hr	No. of animals	Mean, μg %	SE_M	Range
Controls	28	0	—	—
After ACTH (1.5 mg/lb I.M.)				
2	9	0	—	—
After cortisone (10 mg I.M.)				
2	3	26.3	±11.2	11.1–52.8
8	3	11.3	± 1.5	7.3–14.3
24	3	5.3	± 1.7	1.2– 7.9
72	6	0	—	—

centrations returned to zero within 72 hours in all rabbits.

Discussion. The method used here measures all steroids with the 17-hydroxy-configuration, including 17-hydroxycorticosterone, 11-dehydro- 17 -hydroxy-corticosterone, and 11-desoxy- 17 -hydroxycorticosterone (Compounds F, E, and S, respectively), but apparently does not measure other corticosteroids. The finding of negligible amounts of these compounds in the blood of rats, and none in the blood of rabbits is in agreement with the data of Bush(6) obtained by paper-partition chromatography. The absence of circulating 17-hydroxycorticosteroids might be explained either by failure of their production by the adrenal cortex or by their rapid disappearance from the circulation. The injection of ACTH in rabbits failed to produce measurable amounts of 17-hydroxycorticosteroids in their blood. On the other hand the injection of cortisone produced high concentrations, which only slowly returned to zero. These responses suggest strongly that the absence of 17-hydroxycorticosteroids in these animals is to be explained by failure of secretion of these hormones by the adrenal cortex, rather than their rapid disappearance.

Since 17-hydroxycorticosteroids, as determined by this or other presently available methods, do not exist in significant amounts in the peripheral blood of rats or rabbits these animals cannot be used for studies of these steroids. Guinea pigs, however, appear to have amounts of these steroids readily measured by the method and thus are acceptable experimental animals for such studies.

Summary. 1. Quantitative determinations of 17-hydroxycorticosteroid concentrations in the peripheral blood of rabbits, rats, guinea pigs and human subjects were made. 2. Considerable species differences in these concentrations were noted. These differences appear to be quantitative as well as qualitative. 3. Of the species observed, the guinea pig appears to be the best experimental animal for studies of 17-hydroxycorticosteroids.

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Comparative Activities of Thyroxine and its Monosodium and Disodium Salts. (19981)

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Alleviation of the symptoms of thyroid disfunction has been accomplished in the past by the use of thyroid extracts, desiccated thyroid gland, and to a much lesser extent by the use of the pure thyroid hormone, thyroxine. These substances have as a general rule been administered by mouth. More recently, with the development of more effective methods for the synthesis of thyroxine, consideration is being given to the more widespread use of the pure hormone. The advantage to be gained by the use of the pure material is that the drug is a uniform, chemically pure compound, which can be standardized by chemical and physical methods. An apparent disadvantage, based on thyroxine content, is that the thyroxine of desiccated thyroid is apparently better absorbed when given by mouth than is the pure synthetic hormone. Some confusion exists in the literature relative to the comparative effectiveness of free acid thyroxine and its monosodium and disodium salts. It has been reported that in the human, monosodium salts of thyroxine were less effective orally than when given subcutaneously or intravenously (1). It has also been reported that synthetic thyroxine (free acid) was less active when given orally than the monosodium salt (2,7,8). The absorption of crystalline thyroxine from the intestine of the chick was found to be about 20% while the sodium salts were absorbed to the extent of approximately 45% (3). The use of thyroxine tagged with radioactive iodine indicated that about 70% of an oral dose of sodium DL-thyroxine is taken into the blood stream from the intestine (4). Another investigator found that sodium DL-thyroxine has only one-half the activity orally as compared to its parenteral activity (5). In the cow DL-thyroxine was found to be only 12½% as effective orally as when given subcutaneously (6).

Considering the reports that when thyroxine compounds are given by mouth, or parenterally, activity differences occur, which are supposedly dependent upon the route of administration, it seemed advisable to re-evaluate the activity from this standpoint. It also seemed to be important to determine the comparative activities of the synthetic compounds, L-Thyroxine (free acid), monosodium-L-Thyroxine,* and disodium-L-Thyroxine when given by different routes. These compounds were administered by gavage, and by subcutaneous injection, and the data are here recorded.

Materials and methods. Young adult male rats of the Sprague-Dawley strain, weighing approximately 110-120 g, were used as the test animal. All rats were maintained on a standard 18% casein diet during the experimental period. The animals used in each assay were divided into 3 major groups: 1) Normal, untreated controls, 2) Propylthiouracil controls, and 3) Experimentals. All animals were weighed daily, and the average weight changes calculated at the termination of the test period. *Propylthiouracil* was administered according to the technic of Dempsey and Astwood (9). Thirty milligrams of the antithyroxine compound was added to each 100 g of diet. The diet was offered *ad lib.*, and the average food consumption per rat was approximately 225 g in 14 days. Calculated from the average food consumption records, each animal received 67.5 mg of propylthiouracil during the assay period.

Solutions of L-Thyroxine and disodium-L-Thyroxine were prepared from pure crystalline monosodium-L-Thyroxine. Dosages were in $\mu g/100$ g of body weight. All dose levels of each of the 3 Thyroxine compounds were administered to two experimental groups, one group receiving the Thyroxine by gavage and the other by subcutaneous injection. A single stock solution of the required concentration was used for both routes of administration. The syringes used for the injections were 0.25 cc graduated in 0.01 cc, and the needles were No. 27 gauge. Gavage was given with a No. 8 French catheter. The animals received treatment for 14 days and were killed on day 15.

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* Syntheroid®, Travenol Laboratories.

ORAL ADMINISTRATION
OF THYROXINE IN THE RAT

— •— FREE ACID THYROXINE
---o--- MONO-SODIUM SALT OF THYROXINE *
--·+-- DI-SODIUM SALT OF THYROXINE

* BAXTER'S NA-L-THYROXINE (SYNTHROID)

FIG. 1.

SUBCUTANEOUS ADMINISTRATION
OF THYROXINE IN THE RAT.

— •— FREE ACID THYROXINE
---o--- MONO-SODIUM SALT OF THYROXINE *
--·+-- DI-SODIUM SALT OF THYROXINE

*·BAXTER'S NA-L-THYROXINE (SYNTHROID)
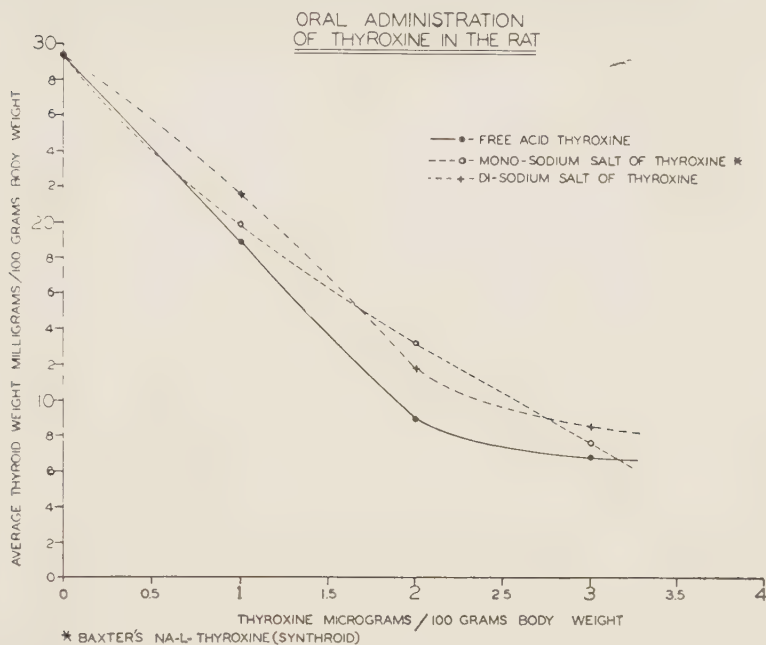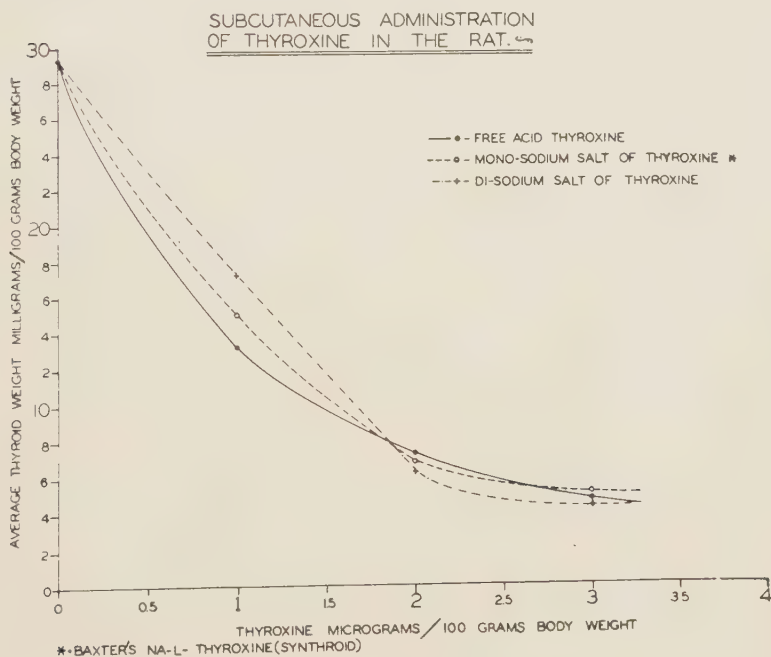
FIG. 2.

The thyroid glands were carefully removed by dissection. The average weight of the thyroids and their average weight per 100 g of body weight was calculated for all the groups in each experiment.

Results. A total of 570 rats were used in the 6 experiments here reported. The average weight of the thyroids of untreated controls was 6.0 μg/100 g of body weight, while those from animals treated with propylthiouracil averaged 29.4 μg/100 g of body weight. A graph of dose-weight relationship when the

thyroxine compounds are given orally, is shown in Fig. 1. It can be seen that to inhibit the effect of PTU, the strength of the dose necessary is 2.5-3.5 μg/100 g of body weight. Fig. 2 is the dose-weight ratio when the thyroxines are given subcutaneously. The amount of the hormone required to maintain the weight of the glands within a normal range is 2.0-2.5 μg/100 g of body weight which is somewhat lower than when the compounds are given orally.

Discussion. The data for the most part are self-explanatory, but two points need further consideration. The first is that oral administration requires a larger dose of thyroxine to bring the propylthiouracil treated thyroid weights into line with normal weights than do subcutaneous injections of the same compounds. Adsorption of the thyroxine in the intestine is a factor in dose requirement (Fig. 1 and 2). The thyroid weight in most of the groups shows a large standard error. Despite the large standard error values, further statistical analysis shows that these values are not too significant because of the great differences between the mean thyroid weight of the control as compared to the mean thyroid weight of the experimental groups. This fact is further emphasized in that the P values for all the groups are less than 1 x 10^{-4}. A P value of 5 x 10^{-2} is statistically significant in most experiments.

Summary and conclusions. Experiments, using 110-120 g Sprague-Dawley male rats, were conducted to compare the activity of L-Thyroxine, sodium-L-Thyroxine and disodium-L-Thyroxine administered by subcutaneous and oral routes. There are no significant differences in the antipropylthiouracil effect of the three synthetic thyroxine compounds when administered by the same route. Considering that the gavage method permits some technical error, the amount of thyroxine necessary to inhibit the effect of propylthiouracil is approximately 50% more by the oral method than that necessary by subcutaneous injection.

Addendum. Since this paper went to press, a report of clinical work with sodium L-thyroxine has been published (Salter, William T., and Rosenblum, Ira, *Am. J. Med. Sc.*, 1952, v224, 628). An assimilation by the patients of 67% of the orally given sodium L-thyroxine was reported.

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Effect of Arginine on Growth and Formation of Arginine Dihydrolase in *Streptococcus faecalis*.*† (19982)

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Gale(1) showed in strain ST of *Streptococcus faecalis* that cells formed during the

first few hours of incubation on an arginine "rich" medium contained a more active arginine dihydrolase enzyme system than cells formed at any later stage of the growth cycle. However, Slade and Slamp(2) found in strain D10 of *S. faecalis* that the first formed cells possessed very little dihydrolase activity. Enzyme formation in this strain commenced after

TABLE I. Growth and Dihydrolase Formation by ST on 'Low' Arginine Media.

Time, hr	Qco$_2$*	% arginine removed	% glucose removed	Cells, dry wt/100 ml	E units†	pH
4	210	69	10	3	630	7.5
5	138	94	19	17	2340	7.4
6	131	99	33	31	4060	7.2
8	70		62	61	4270	6.5
12	50		95	82	4100	6.6‡
25	45		96	80	3600	6.6

* Determinations made with l-arginine as substrate.
† E units = Qco$_2$ × dry wt cells/100 ml medium.
‡ pH adjusted from 5.8 to 6.6 with N NaOH at 10.5 hr.

a short lag period and increased progressively during the growth of the culture. The formation of arginine dihydrolase by this organism was typical of an adaptive process. It is the purpose of this investigation to examine the process of formation of the dihydrolase enzyme system in these 2 strains.

The complete dihydrolase reaction in cell-free extracts of *S. faecalis* occurs in at least 2 steps with citrulline as an intermediate (Slade)(3). 1) arginine + $H_2O \rightarrow$ citrulline + NH_3; 2) citrulline + $H_2O \rightarrow$ ornithine + CO_2 + NH_3. The dihydrolase enzymes will be referred to as (a) arginine desimidase and (b) citrulline ureidase(3).

Materials and methods. Procedures for the culture of the organisms, preparation of cell-free extracts, chemical determinations, and estimation of enzyme activity have been given (2,3). Strain ST was obtained from E. F. Gale of Cambridge University. The chemically defined medium of Slade *et al.*(4) was employed where indicated; however, the concentration of salts A was increased 3 times. The "low" arginine medium consisted of 0.4% casein digest (Difco Casitone), 1% yeast extract (Difco), 0.6% glucose, and 1% Na$_2$HPO$_4$. The "high" arginine medium was the same as the "low" arginine medium except that it contained an additional 0.7% arginine. l-Arginine • HCl, dl-ornithine • HCl, and dl-citrulline were used in all cases. They were obtained from commercial sources. Citrulline was also synthesized from dl-ornithine by the method of Kurtz(5). The rate of arginine desimidase has been shown (3) to be approximately the same as the rate of citrulline ureidase in strains D10 and ST of *S. faecalis* when grown on arginine. There-

fore in these experiments, manometric measurements of the rate of CO$_2$ evolution from arginine are a measure of the complete arginine dihydrolase enzyme system. Rates of ureidase activity in cells grown on citrulline were made with citrulline as substrate.

Results. Growth requirement for arginine. Strains ST and D10 showed an absolute growth requirement for arginine. No significant growth was obtained in the absence of arginine on extended incubation and repeated transfer in the defined medium. However, citrulline, the intermediate compound in the breakdown of arginine, was able to replace arginine in both strains. Total growth in the presence of citrulline was approximately one-fourth that obtained with arginine.

Enzyme formation on "high" and "low" arginine media. Slade and Slamp(2) have shown with D10 that it was necessary to add arginine to a casein digest medium in order that significant dihydrolase formation occur. In the absence of added arginine no significant quantities of enzyme were formed at any stage in the growth cycle. Table I shows that the ST cells formed during the first 4 hours of growth on "low" arginine media possessed high dihydrolase activity (Qco$_2$ 210). A similar result was obtained on "high" arginine medium. Results on the latter medium confirm the observations of Gale(1). A pronounced decrease in enzyme activity followed the disappearance of arginine in both cases. Both media had been largely depleted of arginine in an incubation period of 5-7 hours. Each strain used arginine at approximately the same rate on high arginine medium. A lag period of about 8 hours occurred in strain D10 before utilization of arginine reached the

TABLE II. Effect of Arginine on Dihydrolase Formation by ST in a Defined Culture Medium.

Arginine added, mg/100 ml medium	Cells, mg dry wt/100 ml medium	Q_{CO_2}
1	<2	120
5	<2	150
10	3	160
50	7	150
100	7	203
500	8	204

Cultures incubated 7 hr. Q_{CO_2} determinations made with l-arginine as substrate.

TABLE III. Comparative Activity of Arginine- and Citrulline-Grown Cells.

Activity (Q_{CO_2})			
Whole cells		Cell-free extract	
Arginine	Citrulline	Arginine	Citrulline
Arginine-grown cells			
120	<1	64	52
Citrulline-grown cells			
<1†	<1†	5*	33*

* Culture medium included .1% yeast extract (Difco).

† Activity determined by assay for the presence of citrulline.

Strain ST cells grown on chemically defined medium 16-18 hr. l-arginine was replaced by twice its wt of dl-citrulline.

Q_{CO_2} values of cell-free extracts are per mg dry wt of the extracts.

maximum rate. In ST, however, a lag period of only 4 hours occurred. The Q_{CO_2} of the ST culture decreased between the 6th and 8th hours of incubation to about 60% of its maximum activity. Total growth doubled during the same period. In contrast, enzyme activity in D10 was initially low and increased as growth progressed. Growth was largely completed when approximately 75% of the arginine had been removed. No decrease in enzyme activity occurred during the D10 culture cycle(2).

Quantities of arginine required for enzyme formation. Table II shows that quantities as small as 10 μg arginine/ml resulted in a high level of enzyme formation in young cells of ST on a defined medium. In D10, about 1200 μg arginine/ml medium did not result in significant enzyme formation(2). Consequently, more than 100 times as much arginine was required for the synthesis of a comparable quantity of enzyme in D10 as was required in ST.

Enzyme formation on citrulline. Analysis of cells grown on citrulline offered an opportunity to determine whether arginine desimidase was formed in response to the presence of citrulline as well as arginine. Table III shows that whole cells harvested from a synthetic medium containing citrulline in place of arginine did not show significant arginine desimidase or citrulline ureidase activity. The inability of these cells to metabolize citrulline at an appreciable rate is due principally to lack of permeability of the cell wall to citrulline. This is clearly illustrated (Table III) when whole cells and cell-free extracts from arginine-grown cells are compared. Due to the weak growth response obtained with citrulline it was necessary to add yeast extract to the synthetic medium in order to obtain sufficient cells to prepare a cell-free extract. These extracts did not possess significant arginine desimidase activity (Q_{CO_2} 5). The small value obtained was probably due to the arginine present in the yeast extract. In contrast, the activity on citrulline was 6.5 times that obtained with arginine. Consequently, it was not possible to determine whether cells grown on citrulline possessed arginine desimidase. Also attempts to replace arginine with ornithine plus CO_2(1) in the synthetic medium were not successful.

Discussion. It is possible that the widely differing arginine dihydrolase activity of young cells of strains ST and D10 may be due to a difference in inherited ability to utilize arginine. The lag period required before ST was able to utilize arginine at maximum rate was one-half that required by D10. Also, the weight of cells formed by ST after 6 hours was twice that formed by D10 in the same period. In addition, the total dihydrolase in the former culture was 4 times that present in the latter case.

The relationship of growth to the removal of arginine also differed in the 2 streptococcal strains. In ST the 2 processes occurred concurrently and at a similar rate during the entire culture cycle. The growth of strain D10 preceded the removal of a significant quantity of the arginine present. Ninety per cent of the cells had been formed while only 2.5% arginine was removed. It seems likely in

strain ST that the synthesis of arginine dihydrolase, and consequently the utilization of arginine, is directly connected with growth. In spite of the fact that both cultures require arginine for growth, a highly active arginine dihydrolase enzyme system is not required by D10 for *maximum cell formation* to occur.

A possible explanation of the sharp drop in dihydrolase activity with ST is also evident from these data. The decrease did not begin until practically all the arginine had been removed. Tests for the presence of citrulline during the period of decreasing enzyme activity showed that sufficient citrulline (or a citrulline-like compound) was present to supply the requirement. The cells formed during this period (in the absence of arginine) did not contain arginine desimidase. Consequently, desimidase-active cells formed during the period of arginine fermentation were diluted with desimidase-inactive cells. The total desimidase content (E units) of the ST culture at the start of the phase of decline (6 hr) was the same as the quantity present at the end of the phase (12 hr). The decline in enzyme activity on high arginine media was also due to dilution of the active cells.

The opposite situation existed in strain D10. All cells produced during the culture cycle were formed in the presence of excess arginine. Accordingly, there was no opportunity for ureidase-positive, desimidase-negative cells to be formed.

The formation of arginine desimidase and citrulline ureidase in the presence of either arginine or citrulline as a substrate is in agreement with the concept of "simultaneous adaption"(6).

Summary. 1. Strains D10 and ST of *S. faecalis* were shown on a synthetic culture medium to require arginine for growth. Citrulline, the intermediate compound in the hydrolysis of arginine by these organisms, was found to replace arginine. Citrulline, however, produced only one-fourth as much growth. 2. Strain ST, in contrast to D10, possessed the ability to produce in very young cells a strong arginine dihydrolase enzyme system in the presence of small (10 μg/ml) quantities of arginine. Approximately 100 times as much arginine was required by D10 to produce a comparable enzyme activity. 3. Serial fermentation analyses of cultures of the 2 strains are presented in relation to the rate of synthesis of the arginine dihydrolase enzyme system by the cells. 4. No significant quantities of arginine desimidase were formed when citrulline was used as a substrate for growth. 5. It is concluded that the synthesis of arginine dihydrolase in strain ST is directly connected with growth. Although both ST and D10 require arginine for growth, a strong dihydrolase enzyme system is not required by D10 for maximum cell formation to occur.

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An Activator of Plasminogen in Normal Urine.*   (19983)

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The presence of a potent fibrinolytic agent,

similar to plasmin, in normal urine(1) has recently been confirmed(2). Further investigations have shown, however, that this agent is an activator of plasminogen and is not a fibrinolytic enzyme.

Experiments and results. Activity of urine.
A very high activity was observed when normal human urine, fresh or dialyzed against 0.9% NaCl, was tested by means of our standard fibrin plate method for the estimation of fibrinolytic activity(3). The effect obtained with undiluted urine was comparable to that produced by a solution of crystalline trypsin (Armour, bovine) containing about 15 mg per liter. However, no effect was obtained when the urine was applied to standard fibrin plates in which the adhering plasminogen had been previously destroyed by heating as described by *Lassen*(4). This result suggests that the fibrinolytic agent is an activator of the plasminogen contained in the fibrinogen solution and not an enzyme proper.

Effect of plasminogen. Plasminogen was prepared from human and bovine serum by precipitation with ammonium sulphate(5) followed by dialysis and lyophilization. Samples of plasminogen were then dissolved in the urine and the solutions were tested on heated fibrin plates. A considerable proteolysis was again produced, the magnitude of the effect depending upon the concentration of plasminogen present. A typical experiment with human urine and bovine plasminogen is presented in Fig. 1. Controls with plasminogen alone showed no effect. Confirmation of these results was found in other experiments, where casein was used as a substrate. Thus in one experiment, where 200 mg of a sample of human plasminogen was added to a mixture of 20 ml casein solution (Hammarsten; 4%; pH 7.5) with 10 ml human urine and placed at 39° (with toluene as preservative), the following results were obtained (micro-Kjeldahl):

Zero time: .40 mg N per 2.5 ml
After 4 hr: 1.80 "   " 2.5 "
   " 23 ":   4.17 "   " 2.5 "

The controls (containing urine or plasminogen) were negative (<0.54 mg N after 23 hours).

The following conclusions can be drawn: Urine does not split plasminogen-free substrates (casein, heated fibrin). The addition of plasminogen produces a proteolytic activity which increases with increasing concen-
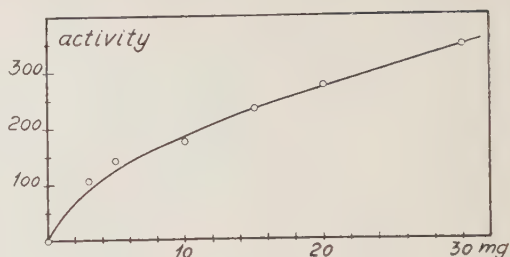
FIG. 1. Proteolytic effect of human urine in relation to concentration of bovine plasminogen. *Substrate:* Standard fibrin plates (.1% fibrinogen; pH 7.8; $\mu = .15$) heated at 85° for 30 min. *Abscissa:* mg plasminogen sample dissolved/ml urine. *Ordinate:* Activity recorded as product (in mm^2) of 2 diameters of the lyzed zones after 20 hr at 37°C (avg of 3 determinations).
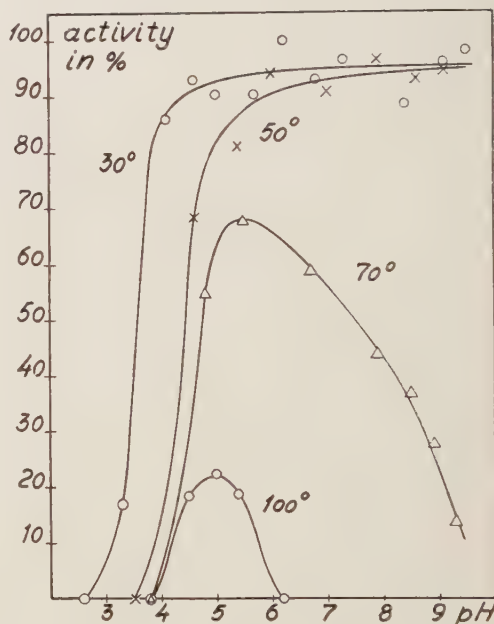
FIG. 2. Fibrinolytic activity of human urine after heating for 30 min. at different temperatures. *Abscissa:* pH of sample during heating. *Ordinate:* Activity in % of unheated control.

tration of plasminogen. Urine apparently contains an activator of plasminogen.

Stability of the urine activator. In order to characterize the urine activator we have in a number of experiments studied its heat stability. Fresh human urine was dialyzed against 0.9% NaCl and heated at different temperatures. Such an experiment is presented in Fig. 2, and it appears that the activator is rather stable at neutral and alkaline reaction but is very easily destroyed at acid reaction.

A dry preparation. Solutions of the urine activator are very labile and the activity often disappears after a few days (even at 0°C). It is therefore necessary to prepare the activator in the dried state. Considerable difficulties were encountered in accomplishing this. In most cases a partly insoluble and denatured product of low activity was obtained. This applies to the precipitation performed by dialysis against slightly acidified distilled water(1). The activator was precipitated by Zn salts at alkaline reaction but could not be recovered from the precipitate. It was not adsorbed or destroyed by $Mg(OH)_2$. Addition of alkali to pH 9 produced a precipitate in the urine, and did not influence the activity of the supernatant. Ammonium sulphate precipitation followed by dialysis against 0.9% NaCl and lyophilization gave soluble products containing about 50% of the original activity. The following procedure was adopted as the most convenient:

To fresh urine 6-N NaOH is added dropwise until pH 9.5 (phenolphthalein). After centrifugation the supernatant is neutralized (litmus), cooled in ice-water and precipitated with 3 vol. ice cold 96% ethanol. After 15 min. the precipitate is isolated by centrifugation, dissolved in a small volume of 0.9% NaCl and dialyzed overnight against 0.9% NaCl (0°C). Insoluble material is removed by centrifugation and the supernatant lyophilized. Yield is about 800 mg per liter urine, containing about 60% of the original activity.

Discussion. The presence of a plasminogen activator in urine is of considerable interest. The urine activator seems to be completely soluble, while the activator present in the tissues (fibrinokinase) appears to be bound preferably to the structural proteins(6-8). The activator present in urine is therefore a physiological plasminogen activator chemically different from the fibrinokinase present in tissues.

Several authors have observed a tryptic agent in urine. *Bendersky*(9) found that some of its properties were different from those of trypsin. Other investigators, however, were not able to find a tryptic enzyme in urine(10) and the problem was finally left unsettled.

Macfarlane(1) recently observed a tryptic agent, which digested fibrinogen and fibrin as well as albumin and casein. The agent resembled plasmin though some differences were observed. The similar properties of the urine factor and plasmin were recently confirmed by our group(2). They find their explanation in the experiments here presented. The effect measured is caused by plasmin formed by an interaction between plasminogen and the urine activator during the estimation. The controversies between the older authors may be explained in the same way though it is not always possible from their descriptions to reproduce accurately the substrates used in their experiments.

It is probable that the presence in urine of a powerful activator of the fibrinolytic system has a definite physiological significance. In view of the importance of a normal excretion of urine, the formation of blood clots in the urinary passages may be just as harmful to the life of the mammalian organism as is a thrombus formation in the blood vessels. The presence of a plasminogen activator in the urine makes possible a rapid resolution of clotted blood in the urinary system. It is not probable therefore that this agent is a simple excretory product. Investigations on normal and pathological cases concerning this problem are under way.

The urine activator must be considered also in an evaluation of the urinary proteases of *Abderhalden* ("Abwehrproteinases"; "Abwehrfermente")(11), which are said to be activated by serum(12). So far no agreement exists concerning the nature—or even existence—of these substances.

In addition to the plasminogen activator we have found small and varying amounts of a trypsin inhibitor in urine. Occasionally traces of a tryptic enzyme were also observed. The potencies of these agents were insignificant in comparison with the activity of the plasminogen activator, and the active substances are probably accidental excretory products.

Summary. 1. The fibrinolytic agent present in normal urine is a plasminogen activator. 2. This activator is very sensitive to acid reaction but rather stable at neutral and alkaline reaction. 3. It is suggested that the signifi-

cance of this physiological activator of plasminogen is to keep the urinary system free from the harmful effects of clotted blood.

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Effect in Man of a New Indandione Anticoagulant.* (19984)

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Since anticoagulant drugs have become an accepted part of the physician's armamentarium there has been considerable stimulus to develop new drugs which give a better anticoagulant effect with less care and expense (1-4). The new anticoagulant drug which is the subject of this study, is an effort in this direction.

Derivatives of 1, 3 indandione were reported to induce hypoprothrombinemia in animals by Kabat, Stohlmann and Smith(5) and a number of clinical studies(6,7) have dealt with the potential usefulness of phenylindandione. A recent report(8) described the development of diphenylacetyl-1,3-indandione (Dipaxin)† in a survey of new indandiones. Correll *et al.* (8) indicate that Dipaxin possessed the unique ability to induce hypoprothrombinemia in rabbits in a quantity 1/200 the amount of Dicumarol necessary to induce an identical hypoprothrombinemia. This would suggest that Dipaxin is the most potent hypoprothrom-

binemic drug available. In order to evaluate the basic properties of this new agent in man and to determine if its potency offered any advantages for anticoagulant therapy the present investigation was undertaken.

Experimental. Prothrombin estimations were made by a diluted plasma technic involving a special heparin-oxalate collection fluid (9). The results are reported in per cent of a dried stable plasma standard which was adjusted to the mean of 20 normal individuals. Ac-Globulin was determined by a one-stage method using Ac-Globulin free plasma as substrate(10). About 75 subjects from the wards of the Los Angeles County General Hospital were used in the control studies in which they were given single doses of the drug; they were unselected as to age and sex. The acutely ill and those suffering from hepatic and renal disease or bleeding dyscrasias were omitted. An additional 35 patients who had indication for anticoagulant therapy received Dipaxin in therapeutic doses. Dipaxin (diphenylacetyl-1,3-indandione) was given orally in the form of 1 mg tablets.

Results. Effect of single doses. Plasma

TABLE I. Effect of a Single Dose of Dipaxin on Prothrombin Levels in Man.

Dose, mg	Control	Prothrombin level (in %) Days								
		1*	2	3	4	5	6	7	8	9
.5	120	123	110	125						
1	107	110	103							
2	122	111	105	113						
4	123	105	93	126						
6	129	113	103	103	130					
8	145	106	86	97	93	106				
10	116	85	78	85	85					
15	131	90	79	77	74	67	90	88	100	
20	121	93	55	55	59	71	72	79	84	83

Each line of figures is an avg of results observed in 4 to 8 patients.

* Actually represents the prothrombin level about 14 hr after drug rather than 24 hr, the drug usually being given at 6 PM, the blood sample withdrawn the following 8 AM.

prothrombin levels were measured at daily intervals in patients given single doses of Dipaxin and these results are summarized in Table I. Six patients receiving only 0.5 or 1.0 mg of the anticoagulant did not show a measurable decrease from the initial level. However, the prothrombin of 4 of 5 patients given 2 mg Dipaxin was slightly but significantly reduced, and a hypoprothrombinemia was established in all of 4 patients given 4 mg Dipaxin. With increasing quantities of Dipaxin a more intense hypoprothrombinemia was established which persisted longer. With a dose of 20 mg prothrombin concentrations were reduced to 55% by the second day and they remained at that level until about the fifth day (Table I). A greater variability in individual response was obtained than is evident in Table I because the figures reported are the average response of a number of patients. A moderate to almost complete resistance to Dipaxin was experienced in one individual in the groups receiving 6, 15 and 20 mg.[‡] In most human subjects given 8 mg or more a detectable hypoprothrombinemia was present within 12 hours, and it was well established in 24 hours. By 48 hours the maximum effect was almost always attained and the hypoprothrombinemia would plateau

‡ Since the present approach consisted of the administration of a fixed dose to all individuals in a group regardless of weight it appeared that a lesser hypoprothrombinemia was induced in obese or very heavy persons.

at about this level for the duration of its effect.

Effect of vit. K upon Dipaxin hypoprothrombinemia. Correll *et al.*(8) have reported that vit. K_1,[§] but not a synthetic vit. K preparation, rapidly restored Dipaxin-induced hypoprothrombinemia in rabbits. Similar studies were performed in human subjects by attempting to inhibit a hypoprothrombinemia before its initiation or to restore to normal a well-established hypoprothrombinemia. Seven patients were given a single dose of 20 mg Dipaxin and simultaneously a dose of 150 mg of a water soluble vit. K analogue, Synkayvite,[‖] was given intramuscularly. The vit. K was then given daily in the same dose until normal prothrombin levels were restored. A protocol of the results in one patient who served as his own control is given in Table II as also is the summary of the data on all the patients treated in the same manner. When 100 mg vit. K_1 was given intravenously to 6 patients with well-established hypoprothrombinemia normal prothrombin levels were rapidly reestablished within 24-48 hours. Two illustrative protocols are included in Table III. Ordinarily normal prothrombin levels would not be reattained for 2 to 5 days. Thus it would appear that vit. K readily counteracts the hypoprothrombinemia induced by Dipaxin.

Use of Dipaxin as a therapeutic anticoagulant. In different regimens Dipaxin has been administered to 35 patients to provide anticoagulant therapy for acute myocardial infarction, suspected infarctions, myocardial insufficiency, pulmonary embolism and thrombophlebitis. In several instances heparin was given during the first 24-48 hours until hypoprothrombinemia was established. Initial studies to obtain basal hypoprothrombinemia (15-30% of normal) utilized first and second day doses of 6 and 4 mg; 6 and 6, 8 and 6, 8 and 8, 10 and 0, 10 and 5, and 10 and 6 mg. With a few exceptions, these conservative doses did not satisfy the

§ Mephyton, a trademarked product of Merck & Co., Rahway, N. J., which is 2-methyl-3-phytyl-1,4-naphthoquinone.

‖ A trademarked product of Abbott Laboratories, North Chicago, Ill., which is 2-methyl-1,4-naphthohydroquinone diphosphoric ester tetra sodium salt.

TABLE II. Protocol of Effect of Synthetic Vitamin K (Synkayvite) on Hypoprothrombinemia Induced by Dipaxin in Humans.

Drug	Control	Prothrombin levels (%) Days											
		1‡	2	3	4	5	6	7	8	9	10	11	12
20 mg Dipaxin*	90	100	28	22	21	24	29	45	80	74	78	80	100
” ” ” + 150 mg vit. K daily (I.M.)*	100	88	68	43	45	72	80	84	94	120			
Same (avg)†	108		64	78	85	112							

* In one patient compared with his own control. † Avg of 7 other patients.
‡ 14 hr ± after drug.

TABLE III. Effect of Dipaxin in Therapeutic Control of Patients Requiring Anticoagulant Therapy.

Patient	Diagnosis	Prothrombin level (in %) Days																	
		1	2‡	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
J.	Arterial occlusion with gangrene	110	100	27	20	16	22	17	21	25	18	25	—	70	—	74	72		
	*	20					2		2			Off therapy							
G.	Myocardial infarction	110	52	22	19	22	25	—	26	38	39	40	34	27	32	20	26	26	
	*	10	5	2	1	2	2	2		3	2	6	3	3	4	2	2	2	
B.	Suspected myocardial infarction	100	49	33	21	21	36	48	52	43	28								
	*	8	6	7	2		3		10			Off therapy							
E.	Thrombophlebitis	120	77	49	31	39	45	43	42	100									
	*	15	10	4	4		6	4	† Vit. K₁										
N.	Myocardial infarction	80	45	18	24	19	20	20	30	25	18	34	—	26	23	25	26	39	
	*	20	4		2	1	1	1	4	2		3		2	2	2	2	4	
M.	Thrombophlebitis	140	82	50	54	39	36	43	39	44	—	32	21	24	60	43	33	90	140
	*	20	10	5	5	3	3	5	4	4		4	2	0	6	4	3	† Vit. K₁	

* Dipaxin in mg.   † Given vit. K₁, 100 mg i.v.   ‡ 14 hr ± after drug.

desired goal of lowering the prothrombin below 50% by the 48th hour. However, when 20 mg was given initially to 12 patients, a satisfactory hypoprothrombinemia was established in 9 of these patients¶ within 48 hours. The prothrombin level at 24 hours usually gave no indication of the subsequent 24 hour fall. If, at 48 hours, the level was less than 50%, further therapy was withheld until the prothrombin level started to rise again. At this time, the following empirical program of determining dosage was used: with prothrombin levels of 50-75%, a dose of 8 mg was given; a level of 35-50% the dose was 6 mg; a level of 25-35% the dose was 4 mg; and

¶ Two of these patients, both with myocardial infarctions and of average weight did not exhibit any hypoprothrombinemia when 20 mg Dipaxin was given. Only after this same dose had been repeated three times at intervals of several days was mild hypoprothrombinemia developed. This relative resistance is as yet not fully understood.

maintenance of therapeutic levels of 15-25%, the dose was from 2 to 4 mg daily. It must be pointed out that these are generalizations based upon the average. Maintenance doses of Dipaxin were also established in six patients who were well-adjusted on Dicumarol therapy requiring levels of drug of 50-75 mg daily. The hypoprothrombinemia was easily maintained with an average daily dose of 3-5 mg of Dipaxin. Several representative protocols are given in Table III. It is apparent that a well-established hypoprothrombinemia being maintained with Dipaxin is dissipated very slowly so that as many as 3 to 6 days may be required for the return of pre-therapeutic prothrombin levels.

Effect on Ac-Globulin. The plasma Ac-Globulin was not decreased by Dipaxin.

Toxic phenomena. Dipaxin produced no uncontrollable hypoprothrombinemia or bleeding in any patient. Several patients complained of nausea with doses of 20 mg but this

was thought to be attributable to the psychic effect of consuming 20 tablets at one time. No other gastric effects were noted. Observations of other objective criteria as the blood NPN, erythrocyte and leucocyte counts and liver function tests did not indicate that Dipaxin produced any change. No delayed toxic reaction was observed in any of the patients who were examined two months after the therapeutic regimen had been discontinued.

Discussion. Dipaxin has a marked hypoprothrombinemic action in man and even in small doses its effect is relatively prolonged. Its effects appear to be relatively predictable and controllable and the hypoprothrombinemia appears to be corrected readily with vit. K. In the limited studies to date it has not produced any bleeding complications or other toxic phenomena. However, these advantages alone do not of themselves suggest that Dipaxin should supplant existing clinical agents.

The ideal hypoprothrombinemic anticoagulant for clinical use should be given orally. The hypoprothrombinemia should be produced rapidly and in a predictable manner involving simple management. Finally, upon withdrawal of therapy normal prothrombin levels should be rapidly restored. Dicumarol is the most widely used anticoagulant of this type and its disadvantages have not been eliminated by several newer anticoagulants (11,12). Dipaxin is considerably more effective in inducing hypoprothrombinemia than Dicumarol in the rabbit(8) and it appears to be roughly 15 to 25 times more active than Dicumarol in man. The general pattern of the curve of hypoprothrombinemia obtained with Dipaxin in man is very similar to that observed with Dicumarol. However, with the doses given, the rate of onset of hypoprothrombinemia appears to be less rapid as well as is the return to normal after withdrawal of the drug. These disadvantages obviously may weigh heavier than any advantage of being able to administer the agent in smaller doses. On the basis of this preliminary study it appears that Dipaxin should be added to the present list of useful anticoagulants. The present studies in man do not suggest that Dipaxin should replace Dicumarol but it is possible that after further trial in these and other clinics other advantages of this new anticoagulant may be revealed.

Summary. 1. A new anticoagulant, Dipaxin (diphenylacetyl-1,3-indandione), has been studied in man. This agent induces an effective hypoprothrombinemia in single doses of as little as 4 mg. It appears to be more potent on a weight basis than any other known agent. After single doses of 20 mg a marked hypoprothrombinemia was usually evident in 48 hours which persisted from 6 to 10 days. Its effects were usually reproducible and predictable. 2. The drug was successfully administered therapeutically. The recommended starting dose is about 20 mg. A schedule of dosages is given. The maintenance of adequate clinical hypoprothrombinemia was obtained with daily doses of 2 to 4 mg. Hypoprothrombinemia was readily overcome with vit. K, the natural vitamin being more effective than the synthetic. No bleeding or other toxic phenomena were encountered. 3. The advantages and disadvantages of Dipaxin as a clinically useful hypoprothrombinemic anticoagulant are briefly discussed.

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Increased Uptake of Iodine-131 by the Thyroid Gland after Administration of Hesperidine Methyl Chalcone. (19985)

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Scott(1) has shown that the rat's thyroid takes up 50 to 200% more radioiodine under the influence of hesperidine methyl chalcone (H.M.C.). It was therefore decided to try this drug in human beings in order to determine its effect upon thyroidal accumulation of radioiodine. This drug is known to suppress the output of I^{131} through the kidney; probably I^{131} stays in the body in circulation a long enough time to favor increased uptake by the gland.

Method. Nineteen patients having the diagnoses shown in Table I were studied. All of these were considered to be euthyroid except K.E. and O.C. who were judged to be hyper- and hypothyroid respectively. Two periods of tests were done on each patient. These were the first or the patient's control period before the administration of H.M.C. and the second or his testing period following the administra-

tion of H.M.C. At the start of the control period, 20 μc I^{131} was given orally. Thyroid gland uptake measurements were done at 24-hour intervals for 96 hours. After a rest period of three days, the same patient's thyroid uptake was again measured for residual I^{131}, then 1.0 g of H.M.C. mixture in water was ingested. After an hour lapse to allow for effective absorption of the drug the patient ingested 80 μc I^{131}. At 24-hour intervals, for 96 hours, his thyroid gland uptake was measured and due correction for decay and effective half-life was made for the residual I^{131} uptake from the control period. The tracer dose of 80 μc I^{131} was large enough to be readily measurable in the presence of residual I^{131} and thus also to minimize statistical differences between the measurements of the two periods.

Results. Studies on the nineteen patients

TABLE I. Percentage Increase of Thyroid Uptake over Normal Following Oral Administration of Hesperidine Methyl Chalcone. I^{131} was administered orally to label iodide pool of body.

Patient	24	48	72	96	Avg increase	Diagnosis
B.B.	16	0	16	19	13.7	Hypertension
M.W.	31		70	34	45	Peptic ulcer
W.S.	41	16	16	21	24	Diabetes mellitus
V.J.	36	20	20	36	28	Inf. hepatitis
K.E.	14	19	28	12	18.3	Hyperthyroidism
O.C.	75	60	18	67	55	Gun shot wound—chest
W.J.	17	8	0	8	8.3	Prob. tbc.
L.J.	18	5			11.5	Gastric ulcer
H.M.	25	29	17	30	25.2	Hepatitis
L.E.	15	24	11	5	13.8	Epilepsy
C.J.	14	30	18		20.7	Hypertension
C.E.		30		5	17.5	Esophagitis
T.G.	12	30			21	Ca lung
Mc G.B.	60	44	32	35	42.8	Rheum. arthritis
U.W.	25	10	24	30	22.3	Hemiplegia
C.D.	9	13	77	50	37.3	Gastric ulcer
W.H.	0	8	0	17	6.3	Arteriosclerosis with myocarditis
G.J.	17	27	41	50	33.7	Diabetes mellitus
O.P.	32	0	4	0	9	Encephalitis
	25.4	20.7	24.5	26.1		
	±2.75*	±3.6	±5.45	±4.67		

* Mean stand. error $= \pm \sqrt{\dfrac{\Sigma \; \text{dev}^2}{n(n-1)}}$.
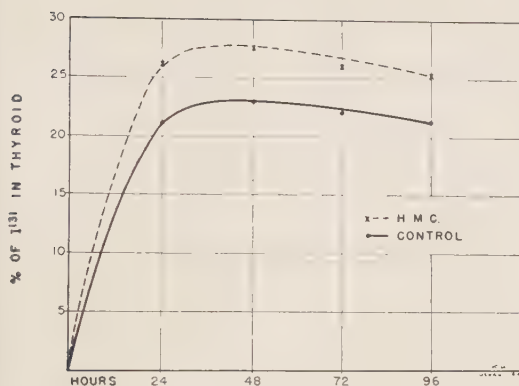
FIG. 1. Average thyroid uptakes on 19 patients before and after hesperidine methylchalcone (H.M.C.) administration using I^{131} as a tracer.

summarized in Table I show that the oral administration of one gram of H.M.C. significantly increases the thyroidal accumulation of I^{131}. This increase averaged between 20.7 and 26.1% at all observed time periods. H.M.C. was more effective in increasing thyroid uptake in some patients than in others. In this study the range was from 6.3% to 55% greater than pre-H.M.C. period when the

uptakes for the four time periods were averaged. The shape of the composite uptake curves when H.M.C. was administered is similar to that obtained before the administration of H.M.C. (Fig. 1). Although it was not possible to obtain hourly or 24-hour urine samples on all patients, they were not significantly different when H.M.C. was administered.

Discussion. These data suggest that the bioflavone derivative, H.M.C., is capable of increasing the thyroidal accumulation of I^{131} in man. These results are consistent with those obtained in rats. In all of the subjects studied, an increase in thyroid uptake was observed. If this drug is used in conjunction with I^{131}, smaller doses might be required to obtain comparable results in thyroid therapy. Such possible usefulness should be evaluated.

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Participation of Adrenal Cortex in Alterations in Carbohydrate Metabolism Produced by Epinephrine.* (19986)

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The subcutaneous injection of epinephrine (0.02 mg per 100 g body weight) into fasted normal rats is followed in a period of 4 hours by a depletion of muscle glycogen and an accumulation of glycogen in the liver(1). Since similar injections of epinephrine also cause a release of adrenal cortical steroids by reason of their capacity to cause ACTH secretion(2), the question arises as to the possible participation of the adrenal steroids in the alterations in carbohydrate metabolism that follow epinephrine injection.

This has been tested by comparing the effects of epinephrine injection in fasted normal and adrenalectomized rats. In addition, since marked differences in the response of these animals was observed, the influence of adrenal cortical hormones in correcting this difference was also estimated.

Methods. Male Wistar rats weighing 180-280 g were used. All animals were kept in a constant temperature room and on a uniform and constant diet. All were fasted for 24 hours before the experiment. Adrenalectomized animals received 1% NaCl as drinking fluid after operation and were used 7-14 days later. Glycogen in liver and muscle was determined by the usual methods, and blood glucose and lactate on samples obtained from

TABLE I.

	No. rats	Liver glycogen*	Muscle glycogen	Glucose in body fluids	Net change
Normal	6	5 ± 2	288 ± 10	30 ± 1	
" + epinephrine	6	48 ± 5	200 ± 19	54 ± 3	
Change		+43	—88	+24	— 21
Adrex.	6	2 ± 5	278 ± 6	22 ± 1	
" + epinephrine	8	8 ± 2	112 ± 11	50 ± 2	
Change		+6	—166	+28	—132
Adrex. + cortisone pellet	2	2	303	30	
" + " " + epinephrine	4	32 ± 5	111 ± 11	47 ± 2	
Change		+30	—192	+17	—145
Adrex. + A.C.E.	5	42 ± 6	286 ± 16	30 ± 2	
" + " + epinephrine	7	92 ± 4	187 ± 9	59 ± 3	
Change		+50	—99	+21	— 28

* All values expressed as mg/100 g body wt.

the tail veins. The carbohydrate levels are expressed in mg/100 g of body weight and for this purpose the muscles were assumed to be 50% of the body weight, and the glucose and lactate to be distributed in 50% of the body weight. All animals received either a subcutaneous injection of 0.02 mg/100 g of a freshly diluted solution of epinephrine (Adrin. Sharpe and Dohme) or a similar volume of normal saline. They were sacrificed 4 hours later under nembutal anesthesia.

Results. (Table I) In normal animals 4 hours after epinephrine injection the loss of muscle glycogen is almost balanced by the accumulation of glycogen in the liver and of glucose in the body fluids. On the other hand, similar injections into adrenalectomized rats caused practically no increase in liver glycogen although the fall of muscle glycogen was approximately twice that found in intact animals. Consequently, the net loss of carbohydrate was some sixfold greater. This was not accounted for by an excessive accumulation of either glucose or lactate in the body fluids since analysis indicated that at the end of the four hour period the levels of both these substances were the same in intact and adrenalectomized animals.

It would appear that in the absence of the adrenal cortical hormones a much larger proportion of the products released by the accelerated rate of glycogenolysis in muscle and liver are utilized by pathways other than those detectable by this technic. These might include an accelerated rate of oxidation, or an increased rate of fatty acid synthesis. Welt and Wilhelmi(3) have brought forward some evidence which indicates that the rate of fatty acid synthesis is increased in adrenalectomized rats.

In this regard the recent experiments of Kerppola(4) are of interest. This investigator has found that large doses of cortisone given to rabbits greatly reduce the phosphorylase activity of liver and muscle, leading in his view to an accumulation of glycogen in consequence of the inhibition of glycogenolysis. If this is correct then in the absence of cortical hormone there would presumably occur an increased rate of glycogenolysis which would be further enhanced by the known stimulation of phosphorylase activity by epinephrine(5).

Since it is known that epinephrine injections of this kind provoke a secretion of adrenal cortical hormones, the effect of maintenance and excess amounts of these steroids on the response of adrenalectomized rats to epinephrine was also studied. Table I indicates that while the previous implantation of cortisone pellets (10 mg) into adrenalectomized rats was followed by a more normal accumulation of liver glycogen, it did not prevent the excessive loss of muscle glycogen and in consequence there is a large deficit in the carbohydrate balance sheet following epinephrine injection. On the other hand, the injection of 1 cc of water extract of the adrenal cortex (Upjohn) every 2 hours for 8 hours before epinephrine injection and for every 2 hours during the period

of epinephrine action led not only to a normal accumulation of liver glycogen, but to a decreased net loss of muscle glycogen. As a result the overall carbohydrate balance sheet was of normal proportions. These results suggest that at least a part of the normal response of the carbohydrate metabolism of fasted rats to the injection of epinephrine is a consequence of the concomitant effect of this hormone in stimulating the release of ACTH from the anterior lobe of the pituitary.

Summary. 1. The subcutaneous injection of epinephrine (0.02 mg/100 g body weight) into fasted adrenalectomized rats is followed by a twofold greater loss of muscle glycogen than in intact rats, and by little or no increase in liver glycogen. Consequently, there is a six-fold greater disappearance of carbohydrate from the body that is not accounted for by the accumulation of glucose or lactic acid in the body fluids. 2. The injection of large quantities of adrenal cortical extract, prior to and during the period of epinephrine action, brings about a restoration of the carbohydrate balance to that found in intact rats.

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Experimental Porphyria. III. Hepatic Type Produced by Sedormid.* (19987)

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An experimental porphyria in rabbits treated with phenylhydrazine, lead, and light has been described in previous reports(1,2). This porphyria was shown to have many features characteristic of the rare erythropoietic (congenital or photosensitive) type of porphyria seen in humans(3). More recently, attempts have been made to find compounds which would produce an experimental porphyria similar to the human hepatic type(3). Ellinger and Riesser(4) described the occurrence of ether-insoluble (uro-type) porphyrins in the urine of patients with trional intoxication, and Fischer and Duesberg(5) later reported the finding of trace amounts of a similar porphyrin in the urine of rabbits treated with sulfonal. Conflicting, and generally negative results have been reported from other laboratories investigating these and similar substances in animals(6,7).

Duesberg(8) reported the presence of porphyria in a patient treated with large amounts of Sedormid (allylisopropylacetylcarbamid). It has now been found that this compound markedly increases the excretion of porphyrins and porphobilinogen in normal rabbits. These and other findings which demonstrate the hepatic nature of this new type of experimental porphyria, form the basis of the present report.

Methods. Rabbits of either sex weighing from 1500 to 3000 g were used, though best results were generally obtained in rabbits weighing less than 2 kg. Sedormid‡ was given orally at the rate of approximately 200 mg per kilo body weight per day in a single dose

TABLE I. Daily Urinary Porphyrin Excretion in Rabbits with Experimental Sedormid Poisoning.

Days of Sedormid admin.	#162 µg/day		#166 µg/day		#167 µg/day		#168 µg/day	
	Uro-*	Copro-	Uro-*	Copro-	Uro-*	Copro-	Uro-*	Copro-
Control	10	23	—	—	—	—	24	36
1	—	—	12	10	16	24	23	38
2	9	33	63	121				
3					17	36		
4	258	75					2050	223
5			1590	329				
6					25	78	—	—
7	12820	240					35250†	384†
8			33150	761	1150	308		
9	32100†	600†	56250	756				
10					13500	447		
12					8030‡	512‡		

24 to 72 hr urine collections combined for determination.
* This includes all ether insoluble uro- type porphyrins.
† These animals were killed before completion of the last 24 hr urine collection. Terminal porphyrin values are therefore expressed in µg/100 ml urine.
‡ This animal died as the result of gastric rupture before completion of the last 24 hr urine collection. Terminal values are expressed in µg/100 ml urine.

or in two divided doses. Attempts made to administer the material in an exact amount parenterally have thus far been unsuccessful because of its relative insolubility in innocuous solvents, including water. The *urine* was collected over 24 to 72 hour periods in metabolism cages permitting separate collection of urine and feces. Porphyrin and porphobilinogen concentrations were determined by previously described methods(2,9,10). Certain modifications employed in some of these analyses will be described in a separate communication. Fluorescence microscopy studies employed a water-cooled General Electric AH6 mercury arc lamp as the light source. One or two Corning filters No. 5-58 (5113) were generally used as primary filters to isolate the 405 mµ line to excite porphyrin fluorescence. An orange Corning filter No. 3-67 (3482) or a light red Corning filter No. 2-63 (2424) was placed between the objective and the eyepiece of the microscope to isolate the fluorescent light.

Results. I. *Urinary porphyrins.* Quantitative data on the excretion of urinary porphyrins have been obtained on 20 rabbits in which treatment with Sedormid was continued for at least 6 days. Peak values for urinary uroporphyrin in these rabbits ranged from 3500 to 60,000 µg per day with an average of

TABLE II. Porphyrin Concentrations in Various Organs and Excreta of 7 Rabbits with Sedormid Intoxication.

Organ or excreta	No. of rabbits studied	μg per 100 g or 100 ml					
		—Uroporphyrin*—		—Coproporphyrin—		— Protoporphyrin —	
		Range	Mean	Range	Mean	Range	Mean
Erythrocytes	5	0- 1.8	.9	tr. - 6.6	1.9	10 - 42	32
Bone marrow	5	0- 1.5	.5	tr. - 14	6.4	15 - 64	42
Spleen	4	0- 13.9	5.8	tr. - 18	7.8	24 - 30	27
Liver, fresh	7	9- 233	108	72 - 167	132	176 - 1700	819
Liver, heated	7	19- 1042	425	65 - 224	139	133 - 1820	585
Kidney	4	30- 120	·76	34 - 162	116	46 - 74	66
Brain	3	0- 1.9	.6	tr. - 2.8	1.6	3.8- 5.5	4.4
Plasma	4	0- tr.	tr.	2.5- 8.1	5.1	5.7- 10.4	8
Bile	5	383- 5480	2122	4600 -39600	19800	13800 -86400	40000
Feces	4	90- 2180	870	1980 - 5900	3560	5500 -12800	9400
Urine	7	13800-76130	24750	260 - 780	490	0·	0

* This includes all ether insoluble uro-type porphyrins.

17,000 μg per day. Peak values for urinary coproporphyrin ranged from 165 to 756 μg per day with an average of 390 μg per day.

Representative data for 4 rabbits treated with daily oral doses of Sedormid are given in Table I. The urinary excretion of coproporphyrin generally rose more rapidly at first, but levelled off after 5 to 7 days. As pointed out previously(2) significant amounts of uro-type porphyrins are excreted in the urine of normal rabbits. From the fifth to eighth day the uroporphyrin values increased sharply to levels sufficient to give the urine a Burgundy red color and a brilliant red fluorescence when exposed to ultra-violet light. Temporary decreases in dose or discontinuation of the drug altogether resulted in a prompt and marked diminution especially in the uroporphyrin excretion.

Spectroscopic analysis (absorption and fluorimetry) of the uro- and coproporphyrin revealed both to be a mixture of free porphyrin and metal complex. The free coproporphyrin was removed by 0.12 N HCl from the primary ethyl acetate extract of the urine. The remaining coproporphyrin was then freed from the metal by extraction with 1.5 N HCl(11). While a marked variation was found in the amount of metal complex, in some samples over 80% of the total was combined with metal, which, on the basis of the absorption spectrum, the complex is probably zinc.

Over half of the total uroporphyrin was excreted in the form of non-fluorescing precursors which developed fluorescence after heating or treatment with iodine.

At least 10 distinct red fluorescing bands were found by calcium carbonate chromatography of the methyl esters. The strongest of these consisted of a uro-type porphyrin crystallizing in long curved hairs with a melting point of 262-265° consisting mainly of type III isomer. Smaller amounts of uroporphyrin I (M.P. ·284°) and of other uro-type porphyrins melting at 242-245° and 252-255° respectively, were also isolated. These and the coproporphyrin were predominantly type III isomers. A detailed report on the physical-chemical properties of the porphyrins will be published separately.

The urinary excretion of porphobilinogen roughly paralleled that of the uroporphyrins. Peak values generally ranged from 20 to 60 Ehrlich units per 100 ml, where one Ehrlich unit represents the color intensity of one mg of urobilinogen.

II. *Tissue, biliary, and fecal porphyrins.* Seven rabbits were killed by cardiac bleeding at the time of marked porphyrinuria. Porphyrin concentrations in various tissues, along with values in bile, urine, and feces are summarized in Table II. Among the most interesting findings were: 1) the high values for liver porphyrins, 2) the increase of liver uroporphyrin on heating, 3) the preponderance of uroporphyrin in urine as compared to bile and feces, 4) the high biliary and fecal coproporphyrin concentrations, 5) the presence of high concentrations of protoporphyrin in bile and feces and its absence from urine, and 6) the relatively high ratio of kidney to urine coproporphyrin as compared to the low ratio

of kidney to urine uroporphyrin.

At autopsy, upon exposure to ultra-violet light, the gallbladder and the liver surface adjacent to the gallbladder, the bile ducts, and the duodenal region around the orifice of the common bile duct showed an intense red fluorescence. This was mainly proto-porphyrin. On cut sections of the liver, the fluorescence intensity of the tissue taken from areas near the porta hepatis, was much stronger than that of tissue taken from more peripheral parts of the liver. However, even in these peripheral areas, pin-point red-fluorescing spots could be recognized which, on fluorescence microscopy, appeared to be within the liver cells of the central half of the liver lobules. On the other hand, fluorescence microscopy examination of bone marrow and peripheral blood smears revealed no red fluorescence.

The porphobilinogen reaction was strongly positive in homogenates of liver and kidney, weak in bile, and negative in bone marrow.

III. *Course.* When the urinary uropor-phyrin excretion reached levels of approximately 7,000 μg per day or more, the rabbits became lethargic and lost their appetite. Transient paralysis of the hind legs and the bladder was observed in many of the animals. X-ray examination showed marked gastric dilatation, constriction of the pylorus and upper small bowel, and distention of the large bowel with gas. Continued administration of Sedormid resulted invariably in sudden death due to rupture of the markedly distended stomach. Undigested Sedormid tablets found among the gastric contents at autopsy may explain the frequent fall in porphyrin excretion during the last day or two of life.

Discussion. The present findings are of special interest in relation to the separation of human porphyria into erythropoietic and hepatic types, evidence for which has recently been accumulated in this laboratory(3). In erythropoietic porphyria one finds large amounts of uro- and coproporphyrin in the bone marrow and circulating red cells, where-as the liver porphyrin concentrations are relatively small. In the hepatic (intermittent acute, cutaneous, and mixed) types of por-phyria, on the other hand, bone marrow and

red cell values are normal, while the liver contains large amounts of porphyrin and por-phyrin precursors in varying combination.

It is evident that the porphyria induced in rabbits by Sedormid corresponds more nearly to the "hepatic" type, because 1) liver porphy-rin concentrations are high whereas bone mar-row porphyrin values are normal, 2) large amounts of porphobilinogen are formed, prob-ably in the liver, and excreted in the urine, 3) a substantial portion of the urinary porphy-rin is excreted as a metal complex, 4) the uro-type porphyrins consist of a mixture of type I and III isomers, however with much more type III than in the human disease, 5) con-siderable amounts of non-fluorescing pre-cursors of uro-porphyrin are present in the urine and in the liver, and 6) the urinary co-proporphyrin is chiefly the type III isomer. Clinically, too, the transient paresis or paraly-sis of the hind legs and the functional gastro-intestinal disturbances in these rabbits are reminiscent of similar findings in patients with the intermittent acute type of hepatic por-phyria.

Studies to be published later have shown a rapid and marked decline in the concentration of liver catalase in these Sedormid-treated rabbits. Since there is a marked accumulation of porphyrins in the liver, this would suggest that Sedormid interferes with the metabolism of the porphyrins and their conversion to iron-porphyrin compounds such as catalase in the liver. It is interesting that no such block is apparent in the formation of hemoglobin in the bone marrow as shown by normal bone marrow and red cell porphyrin values.

Though uro-, copro-, and protoporphyrin concentrations in the liver are all high, their excretory patterns are quite different. The uro- type porphyrins with eight or less car-boxyl groups are excreted chiefly in the urine, the 4 carboxyl coproporphyrin is found in the highest concentration in the bile and feces, and the 2 carboxyl protoporphyrin is absent from the urine and excreted only in the bile and feces. It may be that these excretory patterns are related to an increasing number of COOH groups resulting in increasing water solubility of these porphyrins or their sodium salts. Further studies are required to determine

whether the difference in porphyrin concentration in the kidney is due to differences in tubular reabsorption of uro- and coproporphyrin.

The high concentration of protoporphyrin in the liver parenchyma adjacent to the gallbladder suggests that considerable amounts of this porphyrin are reabsorbed from the gallbladder by way of the lymphatics.

Summary and conclusions. 1. Allylisopropylacetylcarbamid ("Sedormid") produces an hepatic form of porphyria in rabbits. 2. The livers of these animals contain large amounts of proto-, copro- and uroporphyrin, the latter chiefly in the form of non fluorescing precursors. Porphobilinogen is also present. 3. The protoporphyrin is excreted only, and the coproporphyrin chiefly, in the bile, most of the uro- type porphyrins appearing in the urine, in amounts ranging up to 60 mg in 24 hours. The latter consists mainly of type III isomers, but type I has also been identified in small amount. Large amounts of porphobilinogen are excreted in the urine. 4. The porphyrin content of erythrocytes, bone marrow, spleen, and brain is within normal limits. 5. Transient paresis of hind limbs, dilatation of the stomach, and constriction of the pylorus are noted, with irregular small bowel spasm. Death is invariably due to rupture of the stomach. 6. The possibility is discussed that the genesis of the porphyria is related to a primary effect of the Sedormid on the formation of iron porphyrin enzymes in the liver cell.

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Histamine Sensitivity and Anaphylaxis in the Pertussis-Vaccinated Rat.* (19988)

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The intact, white rat is known to be relatively insensitive to both histamine and anaphylactic shock. The resistance to both these insults can be significantly diminished by adrenalectomy(1-3) or hypophysectomy(3,4). This behavior is not unlike that exhibited by the mouse(5,6). In the latter species it has been reported that a preliminary inoculation of *Hemophilus pertussis* likewise diminishes the resistance to both histamine(7) and anaphylactic shock(8). The present report is concerned with the effect of *H. pertussis* vaccination on these insults in the white rat.

Methods and results. (1) Female, white rats of about 100 g weight were injected intra-

TABLE I. Effect of Pertussis Vaccination on the Resistance of the Rat to Histamine.

Histamine phosphate, mg/kg	Mortality rate*	
	Control	Pertussis-vaccinated
187.5	0/10	0/10
375	0/10	
750	0/10	3/10
1500	2/10	13/17

* Mortality rate = number of animals dead after 24 hr/No. animals tested.

peritoneally (i.p.) with 25,000 million phase I *H. pertussis* organisms contained in a volume of 1 ml. On the fourth day after inoculation groups of animals were injected i.p. with various concentrations of histamine diphosphate solution in saline. As controls, unvaccinated animals were treated concurrently. The figures in Table I indicate the mortality rate, determined as the number of animals dead after 24 hr/number of animals tested. It is apparent that *H. pertussis* is effective in lowering the resistance of the rat to histamine.

(2) A series of white rats of approximately the same weight received a single i.p. inoculation of 1 ml of a mixture containing 25,000 million *H. pertussis* organisms and 0.1 ml of horse serum. A control group received only the 0.1 ml of horse serum diluted to a 1.0 ml volume. On the tenth subsequent day both groups were challenged by the intravenous injection of 0.1 ml of horse serum. The results show a mortality rate of 0/27 for the control group as compared to 47/50 for the pertussis-inoculated group. Although the three surviving animals in the pertussis-vaccinated series showed symptoms of severe shock, only an occasional animal in the control group exhibited shock symptoms, always minimal. Thus, it has been shown that pertussis vaccination possesses the capacity of markedly increasing the anaphylactic sensitivity of the rat to an antigen.

(3) Two small groups of rats, vaccinated with pertussis and sensitized to horse serum, were given 25 mg cortisone subcutaneously 4 and 18 hr before challenge, respectively. The mortality rates were 6/6 and 2/5, respectively, thus suggesting that cortisone, in proper doses and at correct time intervals, may have the capacity of protecting the sensitized rat against fatal anaphylactic shock. The surviving animals all exhibited marked shock symptoms.

(4) Three rats of each of 5 experimental groups were sacrificed by asphyxiation with chloroform and immediately autopsied. These groups consisted of (a) normal, untreated animals, (b) animals inoculated with pertussis vaccine and sensitized to horse serum 10 days previously, (c) animals treated as in (b) but sacrificed during anaphylactic shock, (d) animals in histamine shock after pertussis vaccination and (e) unvaccinated, horse serum sensitized animals following challenge with horse serum. No pathological post mortem findings, gross or microscopical, were evident in rats of groups (a), (b) and (e). The animals of group (c) showed grossly, right heart dilation, collapsed lungs, congested liver, gut and brain and essentially normal adrenals and spleen. The gross post mortem findings of the animals in group (d) showed dilation of the right auricle, markedly hemorrhagic and somewhat collapsed lungs, markedly congested liver and brain and essentially normal adrenals and spleen. The microscopical changes of the latter two groups were strikingly similar. The vessels of the heart were distended with blood cells; there was some vacuolization with separation of the muscle fibers. The lungs were congested and there were areas of atelectasis, edema and dilated alveoli. Occasionally, about the bronchi was seen an infiltration of cells. The liver showed minimal congestive changes. The brain was edematous with meningeal congestion. There was marked congestion of the medulla of the adrenals with minimal cortical congestion. The spleens were essentially normal.

Summary. Inoculation of the white rat with *Hemophilus pertussis* increases its sensitivity to histamine. Pertussis mixed with horse serum markedly enhances the ability of the antigen to produce anaphylactic sensitivity.

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Mechanism of Anaphylaxis in the Rabbit. Further Evidence Against Plasma Protease Mechanism.* (19989)

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The theory that the development of anaphylaxis depends upon the activation of plasma protease (plasmin, or fibrinolysin) has numerous adherents. Although this theory lacks the support of direct and unequivocal evidence, and in spite of direct evidence in opposition(1), it enjoys sufficiently wide acceptance to be indicated as the probable mechanism of anaphylaxis in a recent review(3). The literature pertinent to the activation of plasma protease by immune reactions was recently reviewed briefly by Geiger(4), who, as a result of his own careful experiments, considered it "unlikely that anaphylactic symptoms in rabbits can be attributed solely to activation of plasma protease." In our studies of the mechanism of the anaphylactic reaction in the rabbit, we have utilized as a criterion the release of histamine from blood cells, which follows the addition of antigen to the blood from a sensitized rabbit *in vitro*. In an earlier publication(1), we indicated that the participation of plasma protease in the *in vitro* release of histamine in rabbit blood was doubtful because: 1. Excessive quantities of soybean trypsin inhibitor failed to inhibit the histamine release, and 2. Large amounts of bovine fibrinolysin failed to release significant amounts of histamine.

To obtain an even more direct type of evidence, we have now activated plasma protease of rabbit blood by means of streptokinase both *in vitro* and *in vivo,* and have compared the effects of antigen and streptokinase with respect to the activation of protease, the release of histamine *in vitro,* and the development of anaphylactic symptoms *in vivo*. The results of the *in vitro* experiments, Table I, indicate that there is a complete lack of correlation between histamine release and protease activation. Homologous antigen released a large amount of histamine and failed to activate a detectable amount of plasma protease; conversely, streptokinase markedly activated plasma protease and in so doing did not release significant amounts of histamine. From the data in Table II, it is apparent that there is no correlation between protease activation *in vivo* and the development of anaphylaxis. There is no significant difference in protease level between the blood of normal rabbits and blood withdrawn from challenged rabbits at the time of anaphylactic death, prior to death, or during severe anaphylactic symptoms. Conversely, rabbits treated with streptokinase showed no anaphylactic symptoms even though there was a several-fold elevation of the plasma protease level in all cases but one. The evidence presented here not only casts extreme doubt upon the plasma protease theory of the mechanism of anaphylaxis in the rabbit, but also calls for a more critical attitude toward this theory in relation to other species.

Experimental. The streptokinase (SK) preparation, kindly supplied by Mr. Frank Ablondi, of Lederle Laboratories, was dissolved in physiological sodium chloride solution immediately before use. The SK units indicated are Lederle units.

The details of rabbit sensitization, and the *in vitro* histamine release technic were presented previously(1,2). The *in vitro* histamine release was carried out both at 37.5°C,

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* A preliminary report was presented at Federation Meetings, New York, March 1952.

TABLE I. Histamine Release and Protease Activation in Sensitized Rabbit Blood *In Vitro*.

	Histamine release,* μg/ml blood				Clot lysis time,† min.			
	1	2	3	4	1	2	3	4
SK 1750 U‡	0	0	0.06	0.29	5, 7	6, 7	5	5.5
875 U	0.19	0	0	0	7, 8	6, 12	6	5
350 U	0	0.13	0	0.27	35	25	7	5.5
Antigen	4.4	1.7	2 3	2.1	No lysis in 3 hr			

* At 37.5°C.
† Time required for complete lysis of the plasma clot at 29.5°C.
‡ Lederle units of streptokinase per ml of blood.

TABLE II. Relation of Plasma Protease Level to Anaphylactic Symptoms.

Non-sensitized animals Untreated Protease units*	Sensitized animals Egg white i.v.†		Non-sensitized animals Streptokinase i.v.‡	
	Protease units	Symptoms	Protease units	Symptoms
4.2	5	D§ 6 min.	27	0
5.8	4	D 30 "	6	0
8.8	13	D 4 hr	24	0
6.6	3	D 1 "	19	0
4.2	8	D 6 min.	43	0
8.6	4.9	Severe, recovered	41	0

* Per 100 ml of plasma.   † 1 ml whole egg white per rabbit.   ‡ 105,000 Lederle S.K. units per 2.5 kg.   § D indicates anaphylactic death.

and at 29.5°C; the results at the two temperatures were essentially the same. The following procedure was used for the protease activation and clot lysis determination *in vitro*: In a 10 x 75 mm test tube, at 29.5°C, 0.3 ml of plasma (from the sensitized rabbit blood) was mixed with 0.2 ml of saline, a saline solution of SK, or a saline solution of egg white diluted 1:100; 0.1 ml of thrombin in 50 percent glycerol was added immediately, and the contents of the test tube were mixed thoroughly. The lysis time was taken from the time the thrombin was added until the clot appeared completely fluid. Lysis time determinations were run in duplicate, and when the duplicates did not agree both results are indicated. The clot lysis was demonstrated more readily at 29.5° than at 37.5°C. At the higher temperature, the SK-treated samples showed a rapid partial lysis which stopped short of complete lysis, perhaps because of a rapid inactivation of the protease at the higher temperature. In the *in vivo* experiments, blood samples were withdrawn by cardiac puncture into syringes containing 1 ml of M/10 sodium oxalate for every 7 ml of blood. Blood was withdrawn 10 minutes after the i. v. administration of SK or egg white, except in cases where the animals died in less than 10 minutes. In such cases, the blood was taken just as the animal appeared to be dying. Blood cells were removed by centrifugation, and protease was isolated from the plasma by the method of Ungar(5). Quantitative protease determinations were done by the method of Geiger(4), and the results are expressed in terms of the unit defined by Geiger.

Summary. The plasma protease theory of the mechanism of anaphylaxis in the rabbit appears untenable because: (1) Homologous antigen will release histamine *in vitro* or will produce fatal anaphylaxis *in vivo* without significant activation of plasma protease. (2) Streptokinase will activate plasma protease both *in vitro* and *in vivo* without producing any symptoms of anaphylaxis *in vivo* or releasing any histamine from the blood cells *in vitro*.

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Thyroid in Pulmonary Injury Induced by O_2 in High Concentration at Atmospheric Pressure.* (19990)

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Hypophysectomy has been found to afford a very appreciable measure of protection against the pulmonary damage which O_2 at high pressure of several atmospheres (OHP) (1) and in high concentrations at atmospheric pressures(2) inflicts in animals exposed thereto and there is very convincing evidence that such protection is in part at least due to the elimination of adrenocortical influences consequent upon the absence of the adrenocorticotropic substances(2,3). Thyroid activity is diminished in hypophysectomized animals and since the adverse effects of exposure to OHP are so intimately related to metabolism especially to the production and accumulation of CO_2 in the tissues, it was of interest, to determine what effect the administration of thyroid might have on the adverse pulmonary reaction to O_2 in high concentration at atmospheric pressure in both normal and hypophysectomized animals. To this end the experiments described below were carried out.

Procedure. Young male albino rats of about 120 g body weight were used as experimental animals. The feeding of desiccated thyroid (20 mg per rat per day) was begun 7 days prior to and continued during the period of exposure. The exposures to O_2 in concentrations of 94% and above at atmospheric pressure were made in a chamber which provided for the continuous absorption of CO_2, which as analyses showed did not exceed 0.1% except in one instance when it rose to 0.3% for a short interval. The temperature was maintained at that of the room or slightly above. A control group of normal and hypophysectomized animals was maintained under identical conditions in the air at atmospheric pressure. The criteria of the adverse effects of the increased O_2 was the general behavior of the animals, their survival times and the post mortem macroscopic pathological changes

in lungs, thorax and liver.

Results. In the first series of experiments in which 11 animals were exposed, it was found that the non-hypophysectomized animals which had been fed thyroid were the first to show ill effects; they became hyperpneic and lethargic at about the 20th hour of exposure. These symptoms increased to dyspnea and coma and the animals succumbed at about the 46th hour. The next to succumb were the non-hypophysectomized animals which had been fed no thyroid; the symptoms in these were essentially the same as those of the thyroid fed animals but their onset was somewhat later and they succumbed at about 56 hours. These data indicate, therefore, that thyroid had distinctly augmented the susceptibility of the normal rats to the adverse influence of the increased concentration of O_2 at atmospheric pressure. The third in order of onset of symptoms and death was the group of hypophysectomized animals which had been fed thyroid. They succumbed at an average of 66 hours of exposure. By far the most resistant were those hypophysectomized animals which had been fed no thyroid; only a part of these reached a severity of terminal states at the end of 96 hours of exposure at which time all were sacrificed. These procedures were repeated in a second experimental series of 13 animals. Seven hypophysectomized and four non-operated animals were exposed to the increased O_2 and controls for these were exposed to air at atmospheric pressure. The separation of the effects on the various groups was not quite as sharp as in the first experimental series but the results were in essential agreement therewith.

In both of these experimental series the post mortem examination of the thoraces, lungs and livers of animals which had succumbed to the increased O_2 or were sacrificed in terminal states revealed in various degrees the typical macroscopic changes described elsewhere as result of increased O_2(2,3);

massive hydrothorax with a clear, watery, bloodless solution which clotted on standing, pulmonary congestion, hemorrhage, oedema and hepatization so that the lungs frequently sank in fixing solution; the liver was enlarged and congested as were also the adrenals in the non-hypophysectomized animals. In the non-operated animals all of these changes were markedly more severe in the thyroid fed animals than in those not having been fed thyroid. Similarly, in the hypophysectomized animals, thyroid feeding enhanced the susceptibility to these pathological effects so that it approximated that of the normal non-thyroid fed animals, whereas the hypophysectomized animals not receiving thyroid remained relatively free of the more severe changes. Clearly, thyroid feeding augments the susceptibility to the pulmonary damaging effects of increased O_2 in the normal animal and counteracts to large extent the protective action which hypophysectomy affords against these effects.

Discussion. The importance of CO_2 as a causative agent in the reaction of animals to O_2 at high pressure is well recognized(4-9) and the disruption of the carriage of the CO_2 by the hemoglobin in such situations results in an elevation of tissue and blood CO_2 and an increased acidity(5,10). It is held by some authors(11,12) that the cause of the pulmonary damage by O_2 at atmospheric pressure is an elevation of blood CO_2. If this latter be so, the effects of thyroid feeding seen in our present experiments might simply be explained as being due to an additive effect of a further CO_2 elevation in tissue and blood as a result of increased O_2 metabolism. But the small elevation in blood CO_2 would, by itself, appear to be insufficient to fully explain the pulmonary damage. It is highly probable that increased O_2 has an adverse effect of its own and renders the tissue peculiarly susceptible to CO_2 changes.

Taken as a whole the experimental evidence points to a multiplicity of factors which contribute to the over-all picture of pulmonary damage, hydrothorax and liver involvement induced by exposures to O_2 in high concentrations at atmospheric pressure and calls for further investigation.

Summary. 1. In a study of the influence of O_2 in high concentrations (94% at atmospheric pressure) it was found that the administration of desiccated thyroid augments the adverse effects of O_2 on normal and on hypophysectomized rats as shown 1) by a shortening of both the onset of the symptoms—hyperpnea, lethargy, dyspnea, coma—and the survival times, and 2) by increasing the severity of macroscopic *pathological changes,* including massive pleural effusion and hydrothorax, congestion, oedema and hepatization of the lungs and enlargement and congestion of the liver. 2. The pronounced protection which hypophysectomy provides against the adverse pulmonary effects of increased O_2 is in large degree counteracted by the administration of thyroid to the hypophysectomized animal. This protection is, therefore, attributable not only to the loss of adrenocortical principles but also to the loss of thyrotropin. The possible involvement of an increased CO_2 in the thyroid effects is discussed.

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Calcification IX. Influence of Alkaline Earths on Survival of the Calcifying Mechanism.*† (19991)

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The purpose of this investigation was to study the influence of divalent cations on the survival of the mechanism responsible for calcification *in vitro* of hypertrophic epiphyseal cartilage. This was undertaken as an extension of our recent studies which indicate the existence of a constituent (or system) responsible for the disposition of cations(1-4). That some cations may influence the survival of the calcifying mechanism was already indicated in our earlier studies of strontium rickets, in which it was stated that "it can be postulated that strontium in some manner prevents the destruction of the calcifying mechanism"(5). Such a possibility was also indicated from recent studies where, by means of cations, the reversible inactivation of calcification *in vitro* was demonstrated(1,4).

In the present study calcification *in vitro* was determined following incubation in basal salt solution containing alkaline earth and other divalent cations. The results were compared to those obtained following incubation in basal salt only, in which there is a progressive decrease of the calcifying mechanism(6,7).

Experimental. The bone cartilage sections were sliced from the tibiae of Wistar rats that had been placed at the age of 22-24 days on a rachitogenic diet(4) for 14-21 days. To insure equal exposure of both sides of the slices, they were suspended in the test solutions in 25 ml Erlenmeyer flasks from glass hooks attached to lightly paraffined cork stoppers. The stopper was kept in place by a rubber band (ca. 3 mm wide to prevent rolling) run along a groove on the upper side and doubled under the flask back to the groove. In each experiment, at least one of

the four slices obtained from each animal was used as a calcification control, and a second as a basal salt solution control. The degree of calcification of the silver stained sections was evaluated with a binocular microscope under 60x magnification, and graded according to the scale described previously(8), and illustrated in the Transactions of the Josiah Macy Foundation(9). The basal salt and calcifying solutions were prepared from stock solutions as described before(2,4), with the exception that dilutions were made with carbonated distilled water rather than with water followed by CO_2 treatment. Such solutions, initially below pH 7, were adjusted to pH 7.3 by swirling vigorously in a wide mouth Erlenmeyer flask. After basal treatment the sections were rinsed with distilled water and transferred to calcifying solution. All incubations were carried out without shaking at pH 7.30 ± 0.05 in a thermostatically controlled room whose temperature was set at 36.5 ± 0.5°C. *Basal Sol.* 70 mM NaCl, 5 mM KCl, and 22 mM $NaHCO_3$/liter. *Calcifying Sol.* Basal components plus 10 mg % Ca^{++} plus 5 mg % P as inorganic phosphate; Mg^{++} absent unless otherwise indicated.

Results and discussion. As seen in Table I, the loss of calcifiability of bone cartilage was markedly retarded by small amounts of calcium. Essentially similar results were obtained with strontium, although the mineral deposits were often not as well delineated as with calcium. The protective action of these two ions persisted for about 11-12 hours, whereas the basal salt controls underwent major loss of calcifiability in 7-8 hours and complete inactivation in 12 hours under our experimental conditions. For short periods of preliminary treatment the differences could be demonstrated when the subsequent periods of calcification were also short, generally of 4-6 hours duration, while the presence of the calcifying mechanism in sections soaked for a longer time could be better demonstrated

TABLE I. Survival of Calcifying Mechanism in Basal Salt Solution Containing Divalent Cations. pH 7.3, 36.5°C.

Added components*	Conc., mM/l	Incubation time, hr	Subsequent period of calcification, hr	Basal + added comp.	Basal	Control
Ca++		3	4	1.7(++++)	1(++)	2.3(++++)
		4	11	2(++++)	1.5(++++)	2.5(++++)
	2.5	7	4	1.9(++++)	0(0)	2.2(++++)
		7	5	2(++++)	1(+)	2(++++)
		13	12	0(0)	0(0)	2(++++)
		14	24	1.5(+)	0(0)	2.5(++++)
	0.625	8	13	2(++++)	1(++)	2(++++)
		11	22	1.5(++++)	1(++)	2.3(++++)
Sr++		3	4	1.5(+++)	1(++)	2.3(++++)
	1.14	7	5	1.5(++++)	1(+)	1.7(++++)
		7	12	1.8(++++)	1.2(++)	2(++++)
		18	18	0(0)	0(0)	2(++++)
	0.285	8	13	1.5(++++)	1(++)	1.8(++++)
		11	22	1.2(+++)	1(++)	2.3(++++)
Mg++		7	12	1(+)	1(+)	2(+++)
	1	7	12	1.6(++)	1.5(++)	3.3(++++)
		9	14	1(+)	1(+)	1.5(++++)
		4	4	0(0)	0(0)	1.5(++++)
	4	4	16	1.2(+++)	1(+++)	1.5(++++)
		6	13	1(+++)	1(++)	2(++++)
	10	7	12	1(+)	1(+)	2(++++)
		7	12	1.9(+++)	1.5(++)	3.3(++++)
Ba++		2	6	1(+)	1(++)	2(++++)
	1	6	16	1(++)	1.1(+++)	2(++++)
		9	11	0(0)	0(0)	2(++++)
		3	19	1.4(+++)	1.1(+++)	2(++++)
		4	5	0(0)	0(0)	2(++++)
	5.0	6	17	1.8(+++)	1.6(+++)	2(++++)
		7	14	0(0)†	1(++)	2(++++)
		8	14	0(0)	0(0)	2(++++)
Be++	0.05	2	16	0(0)	1.5(++++)	
Be++ PO4≡	0.05 1.61	2	14	1.3(+++)‡	2(++++)	
Be++ Mn++	0.05 1	2	16	0(0)	1.5(++++)	
Mn++	1	2	14	0(0)	2(+++)	
		1	18	1.2(++)	1.5(++++)	
Co++	1	2	14	0(0)	2(+++)	
		1	18	1.4(++)	1.5(++++)	
Ni++	1	1	18	1.1(++)	1.5(++++)	

* Mn++ and Mg++ were added as the sulfates, Co++ as the acetate, and the other cations as the chlorides. Phosphate was added as the sodium salt.

† A few flocculent white particles were noted on the stems of the glass hooks.

‡ Subsurface deposit.

§ Untreated section from same animal placed immediately in calcifying solution.

by more prolonged incubation in calcifying solution.

Of the other ions studied (Table I), barium had no apparent influence, in spite of the fact that it is a strong inhibitor when placed in calcifying media (unpublished experiments). Magnesium, which is a mild inhibi-

tor of calcification (2-4,10), either had no effect on survival in basal solution or exerted trace protection. It did not interfere with the protective action of calcium when added to the basal medium, but did retard calcification when included in the subsequent mineralizing step (Table II). All of the other ions studied

TABLE II. Survival of Calcifying Mechanism When Tested in the Presence of Magnesium Ion. pH 7.3, 36.5°C.

Preliminary treatment,* 7 hr	Conc. of Mg++ in calcifying solution, mM/l	Degree of calcification, 12 hr
Basal	0	1(++)
+ Ca++	0	1.9(++++)
+ Ca++ + Mg++	0	2.2(++++)
+ Ca++	1	1(+)
+ Sr--	0	1.7(++++)
+ Sr++	1	0(0)

* (Ca++) = (Sr++) = (Mg++) = 1 mM.

were inhibitory, as shown by the fact that after 1-2 hours of immersion in basal solution containing 0.05 mM beryllium or 1 mM manganese, cobalt, or nickel, subsequent calcification did not occur.

When phosphate was present with beryllium ion in basal solution, only partial inhibition of calcification resulted. This finding indicates that studies of the inhibitory effect of beryllium when placed in a calcifying medium containing inorganic phosphate should be re-evaluated(11-13), since inorganic phosphate interferes with the inhibitory effect of beryllium, probably by the formation of some unionized complex(4).

Actually, beryllium is a marked inhibitor of the inorganic mechanism as shown in these studies as well as previous studies of reversible inactivation(4). Further investigation is necessary to determine whether the block by beryllium is due to its binding of some tissue element, e.g., chondroitin sulfate (4,14), or inactivation of an enzyme such as bone phosphatase(11-13,15). The ability of manganese to counter the beryllium inhibition of phosphatase(15) could not be put to a successful experimental test to resolve the phosphatase question, since manganese per se is inhibitory in basal medium.

The marked influence of calcium and strontium ions on the survival of the calcifying mechanism suggests that these ions form a relatively stable compound with an essential ingredient of the calcifying mechanism. Such a suggestion is compatible with earlier studies which have indicated that combination of some local factor with calcium is an integral part of the calcifying mechanism. In this connection, strontium also has an interesting history. It has already been shown that this cation is capable of yielding mineralized deposits from inorganic solutions(6,7), and evidence was presented that this ion blocks calcification by competing with calcium for some ingredient in the matrix essential for the calcifying mechanism. From the present evidence one might suggest a compound related to chondroitin sulfate(4,14), or adenosine triphosphate(16). This explanation would require that the dissociation constant of the postulated complex be small, in view of the low concentrations of calcium and strontium which retard disappearance of the calcifying mechanism. An alternate possibility is that strontium and calcium ions suppress a component or system which destroys or inactivates the calcifying mechanism.

Summary. 1. Rachitic bone cartilage slices which are suspended in basal medium at 36.5°C become slowly inactivated as shown by their subsequent inability to calcify. Major loss of calcifiability takes place in the first 7-8 hours with complete inactivation evident after about 12 hours. In the first 11 hours, small amounts of calcium or strontium exert a marked protective action against deterioration of the calcifying mechanism. Unlike calcium and strontium, comparable concentrations of magnesium and barium have no influence on the survival of the calcifying mechanism. 2. Beryllium in basal salt solution inactivates the calcifying mechanism, which can be prevented in part by inorganic phosphate. Manganese, cobalt, and nickel also inactivate the system when added to the basal salt solution. 3. Evidence to date favors the explanation that strontium and calcium ions protect the calcifying mechanism by forming a relatively stable compound with a component of the calcifying matrix essential for the mineralizing process, although an alternate explanation that these ions suppress a system responsible for inactivation cannot be excluded.

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Effect of Total-Body X-Irradiation on Relative Turnover of Nucleic Acid Phosphorus.* (19992)

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Supplee and Entenman(1) have observed that fasted adult female Sprague Dawley rats, previously subjected to large doses of total body X-irradiation, have larger livers than fasted non-irradiated control rats. This enlargement of the livers was found not to be due to edema or to the excessive accumulation of lipids. The maximum increase in liver mass occurred at approximately 24 hours following 2500r total body X-irradiation. Since the increase in liver size seems to be due to an actual increase in liver tissue, it was believed that this change might be reflected in the synthesis of cytoplasmic pentose nucleic acid (cPNA). It has been postulated by Casperson, Brachet, and Spiegelman(2-4), that the formation of PNA and protein are intimately related. Abrams(5) studied the effect of X-irradiation on the formation of desoxypentose nucleic acid (DNA), PNA, and protein in rat intestine. He observed that with 500r the incorporation of glycine-1-C^{14} into DNA and PNA purines is depressed, while there is no apparent change in the incorporation of glycine into proteins. This study might be interpreted to contradict the earlier observations of Casperson and Brachet. However, since the incorporation of the radioactive precursor was observed at only one time interval (*i.e.*, 48 hours), and since it is not known at which step between precursor and final product inhibition occurs, it cannot be concluded from Abrams' findings that there is no relationship between PNA and protein synthesis.

In the experiment reported in this paper we determined the incorporation of radioactive phosphorus (P^{32}) into rat liver DNA, cPNA, and nuclear PNA (nPNA) 24 hours post-irradiation, at which time the maximum effect of total body X-irradiation on liver weight was observed. We also determined the same nucleic acid fractions in mice exposed to 600r at 2½ hours post-irradiation.

Experimental. Twenty female *Sprague-Dawley* rats each weighing approximately 200 g were given a total dose of 2500r of 250 kv X-rays filtered through 0.5 mm Cu and 1 mm Al at an average dose rate of 22.7r/min. Food was removed from cages just prior to irradiation. Water was allowed *ad lib.*

throughout the experiment. The rats were injected intraperitoneally with approximately 100 μc of $Na_2HP^{32}O_4$ 24 hours after the completion of irradiation and sacrificed with ether $4\frac{1}{2}$ hours later. Eight female *Long-Evans rats* weighing an average of 280 g were given a total dose of 2440r of 215 kv X-rays filtered through 1.1 mm Cu and 1.2 mm Al at an average dose rate of 12.2r/min. Six female Long-Evans rats were used as controls. Food was removed from the cages just prior to irradiation. Water was allowed *ad lib.* The rats were injected intraperitoneally with approximately 135 μc of $Na_2HP^{32}O_4$ 18 hours after the end of irradiation and sacrificed 4 hours later. Thirty-six male A strain mice, weighing an average of 25 g were given a total body X-irradiation of 600r of 215 kv X-rays filtered through 1 mm of Cu and 1.2 mm Al at an average dose rate of 16.7r/min. Twenty-four male A strain mice were used as controls. Food was removed from the cages 24 hours before the animals were sacrificed. Water was allowed *ad lib.* throughout the experiment. The mice were injected intraperitoneally with approximately 20 μc of $Na_2HP^{32}O_4$ 20 minutes after the end of irradiation, and sacrificed 2 hours later.

The livers were removed immediately, weighed and put in iced beakers. The separation of nuclei and cytoplasm and the isolation of the nucleic acids has been described previously(6,7).[†] Essentially it consists of homogenizing the livers in isotonic saline and separating the nuclei and cytoplasm by differential centrifugation. The nuclei were washed in citric acid. The DNA-protein and nPNA-protein were separated in a sodium bicarbonate-sodium carbonate buffer. The DNA was then isolated by a modified Beck and Klein method(7). The nPNA-protein was obtained as a trichloroacetic acid precipitate, and the protein was split from the nucleic acid in cold sodium hydroxide.

The cPNA protein was directly precipitated from the cytoplasm and the cPNA was ob-

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† The mouse livers were homogenized in 0.25 M sucrose according to Schneider and Hogeboom's(8) method. The rest of the isolation procedure for the mouse livers followed the method of Barnum *et al.*(9).

tained in the same manner as the nPNA. After obtaining the crude DNA, it was purified in the following manner. The DNA was dissolved in 0.1 M sodium hydroxide, and 0.2 ml of a saturated solution of disodium phosphate were added. The DNA was then reprecipitated five times with hydrochloric acid and methyl alcohol. At this point the specific activity remained constant and did not change upon further reprecipitation. The specific activity of the PNA-P was found not to change upon reprecipitation, therefore it was reprecipitated only once. To test whether there was any contamination of nPNA with DNA, or cPNA with DNA, or DNA with PNA, the nucleic acids were hydrolized and paper chromatographed. No uracil was found in the DNA fraction and no thymine in either one of the PNA fractions. To verify further the purity of the nucleic acids, the PNA was determined by the orcinol reaction and the DNA by the diphenylamine reaction. The density of the color developed per microgram of phosphorus agreed well with that of commercial nucleic acid preparations(6). The P^{32} samples were counted with a Victoreen-Geiger tube. The samples were counted with an accuracy of at least 2%, except the DNA samples from the irradiated rat livers which counted as low as 25% above background, and therefore their counting error was as high as 14%. The specific activity is expressed as counts per minute per milligram of nucleic acid phosphorus divided by the counts per minute injected, corrected for the weight of the animal.

Results and discussion. As can be seen in Tables I, II, and III the specific activity of the cPNA-P of the livers of irradiated rats and mice is increased in all cases. On the other hand the specific activity of nPNA-P and DNA-P is decreased. The liver weights, taken as per cent of total body weight, are increased 29% in the irradiated Sprague-Dawley rats, while there is no significant difference in the weights of the livers from the Long-Evans rats. At present the difference between the two strains is not understood. The two sets of rats came from different colonies, received the radiation dose at a different rate, and were not quite the same age.

TABLE I. Averages of Specific Activity $\times 10^5$ of Nucleic Acid-Phosphorus and Liver Weights of Sprague-Dawley Rats.*

	Controls	Irradiated	Critical ratio†
cPNA-P	36.4 ±1.4	48.2 ±2.6	4
nPNA-P	97.7 ±4.6	78 ±3.8	3.3
DNA-P	1.90± .23	.28± .026	7.2
Liver wt as % of total body wt	4.12± .07	5.32± .12	8.5

* 20 control rats; 20 irradiated rats.

† Critical ratio $= \dfrac{M_1 - M_2}{\sqrt{(\sigma M_1)^2 \pm (\sigma M_2)^2}}$

M = mean; σM = stand. error of mean.

TABLE II. Averages of Specific Activity $\times 10^5$ of Nucleic Acid-Phosphorus and Liver Weights of Long-Evans Rats.*

	Controls	Irradiated	Critical ratio
cPNA-P	43.7 ±1.34	55.4 ±2.2	4.5
DNA-P	3.35± .69	.57± .05	4
Liver wt as % of total body wt	4.16± .29	4.01± .34	.33

* 6 control rats; 8 irradiated rats.

TABLE III. Averages of Specific Activity $\times 10^4$ of Nucleic Acid-Phosphorus and Livers* of Male A Strain Mice.

	Controls	Irradiated	Critical ratio
cPNA-P	6.8 ± .24	7.4 ± .32	1.5
nPNA-P	76.3 ±16.2	54.2 ±8.6	1.2
DNA-P	.607± .11	.405± .078	2

* 9 livers from irradiated mice were pooled, and 8 livers of the control mice.

Whether these factors have any influence on the weights of the livers is not known.

In preliminary experiments total nitrogen determinations were carried out on liver cytoplasm from four fasted irradiated and four fasted control male Sprague-Dawley rats that were sacrificed 24 hours after exposure to 2500r. The average nitrogen values obtained (corrected for a 200 g rat) were 178 mg ± 7.4 mg for the irradiated rats and 148 mg ± 4.2 mg for the control rats. This represents an increase of 20%. Under the same conditions the incorporation of C^{14} labeled formate into liver cytoplasmic proteins (fat extracted TCA precipitate) was also determined. For-

mate was given intraperitoneally to 4 irradiated and 4 control rats 2 hours before sacrificing the animals. An increase of 34% above the control values was found in the specific activity (counts per milligram of protein). These preliminary findings suggest that the increase in liver weight is due to an increased protein synthesis in the irradiated animals. The simultaneous increase of cPNA-P specific activity is compatible with the concept of an interrelationship of protein and PNA synthesis.

The increase in cPNA-P specific activity and liver weights may be akin to an attempt of the liver to regenerate, after having been damaged by the exposure to X-irradiation, but which cannot be carried to completion due to an inhibition of cell division. From these data it is not known whether the observed increase in the cPNA-P specific activity is due to an increase in the turnover of all the cPNA fractions or whether it is due to a relative increase in the amount of "supernate PNA" which normally has a higher turnover than the other fractions(10,11).

Summary. The incorporation of P^{32} into DNA, nuclear PNA (nPNA), and cytoplasmic PNA (cPNA) of liver was measured in rats which had been exposed to 2500r total body X-irradiation and in mice which had been exposed to 600r total body X-irradiation. It was found that the incorporation of P^{32} into cPNA was increased in all cases, while into DNA and nPNA it was depressed. In the Sprague-Dawley rats an increase in the weights of the liver was observed concurrent with the increase in cPNA specific activity. A possible interrelationship between protein and cPNA synthesis has been discussed.

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Experimental Study on Ectopic Beats Following Intravenous Injection of Veratrine.* (19993)

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Subepicardial injection of veratrum alkaloids (veratrine Merck) in the dog's ventricle causes an ectopic rhythm originating in the area of injection. The activity of the ectopic center, firing regular stimuli, is not disturbed by other activation waves spreading over the heart; the center is protected from other stimuli ("protective" block). Thus, a parasystole with simple interference appears regularly(1). On the other hand, true extrasystoles, that is, premature beats with constant coupling to the preceding "initiating" beat(2) were never seen in these experiments. The occurrence of "extrasystoles" following parenteral administration of veratrine has frequently been reported. Since, in general, arrhythmias caused by topical administration of different compounds or electrolytes closely resemble those which appear following the intravenous administration of these substances, we decided to investigate the occurrence of extrasystoles with fixed coupling following intravenous injection of veratridine, cevadine, and a mixture of veratrum alkaloids in the dog.

Method. Dogs weighing 20-25 kg were anesthetized with nembutal and the heart was exposed as described in previous papers by the authors. The right vagus was severed in the neck in each experiment. The electrocardiogram was continuously recorded in Lead II from the time an arrhythmia appeared to the final onset of ventricular fibrillation. Registration was only interrupted when a regular sinus rhythm or a regular tachycardia appeared. A 0.025% solution of the alkaloids was used in all experiments. The solubility of the alkaloids was enhanced by the addition of alcohol. One-tenth of 1 cc of the solution was injected intravenously and if no response appeared within 2 to 4 minutes the injection was repeated in increasing doses up to 1 cc until an arrhythmia was observed. Arrhythmias were seen after injection of about 1.3 mg of veratridine or veratrine Merck. Double doses of cevadine had to be administered in order to obtain the same effect. If no change of the existing arrhythmia was seen after 2 to 4 minutes an additional intravenous injection of the veratrine solution was given. Finally, after 30-50 minutes ventricular fibrillation appeared in all 14 experiments.

Results. In no experiment did we observe extrasystoles with fixed coupling. There were only ectopic ventricular tachycardias with changing form of the ventricular complexes.

Fig. 1a shows the irregularly formed and timed ectopic beats, which—in the last third of the tracing—change into ventricular flutter and fibrillation. Sometimes a bigeminy was suspected as in Fig. 1b and c but closer analysis showed that we are dealing here with other disturbances. A regular sinus tachycardia exists throughout Fig. 1b. The first few complexes show an elevated RS-T segment, presumably due to the appearance of block areas in the myocardium. The same
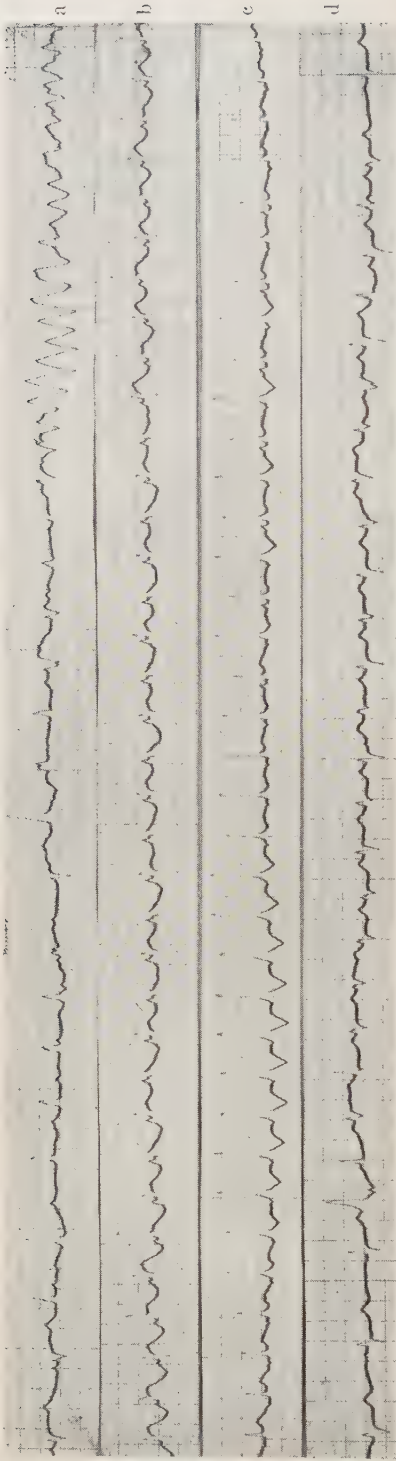
FIG. 1, a–d. The electrocardiographic tracings show different disturbances of ectopic stimulus formation caused by intravenous injection in the dog of veratrine alkaloids. Details are described in the text.

complexes alternate. There is no evidence that every second QRS complex is deformed because of the presence of a ventricular extrasystole appearing late in diastole (combination beat) since the rate of the auricles and ventricles as well as the P-R intervals remains constant. We are dealing with a sinus tachycardia and an electrical alternans due to intraventricular block.

In Fig. 1c a ventricular ectopic rhythm and, later, an auricular bigeminy seem to be present. Closer study of the tracing reveals the presence of an auricular ectopic tachycardia with negative P waves; occasionally, however, positive P waves are seen with every second beat. In some places an abnormal QRS complex seems to follow only the inverted P waves but in other areas the stimuli causing the inverted P waves are conducted normally to the ventricles. We are therefore dealing here with electrical alternation in the auricles and aberrantly conducted ventricular beats but no extrasystoles.

Evidence that the heart in these experiments is under strong veratrine action is afforded by the next tracing. In Fig. 1d a sinus bradycardia appears to be present. Actually a 2:1 block exists. This persisted for about 20 minutes and by an artificial mechanical stimulus applied to the ventricle leading to an extrasystole it could always be transformed into a full rhythm with A-V conduction of every stimulus. Thus a right ventricular extrasystole appears in Fig. 1d after the third sinus beat following a light tap with a probe on the conus of the right ventricle. Immediately the 2:1 block disappears and 23 sinus beats are conducted to the ventricle. The form of the QRS complexes changes due to intraventricular conduction disturbances and a tendency to the appearance of an electrical alternans is present. The P-R intervals also change slightly. The mechanical stimulation was repeatedly applied and each time induced a similar series of conducted beats. The length of these series varied and in some instances numbered 60 beats.

This phenomenon of "deblocking" by an extrasystole has been described only in the veratrinized frog heart(3). It is explained by the fact that the refractory phase of the extra-

picture is seen at the end of the tracing. In the middle of the tracing 2 forms of the QRS

systole is short so that the following sinus beat finds the conduction tissue already fully recovered and is conducted to the ventricle. Since this conducted beat appears after a short diastole it also leaves a short refractory phase so that the following beat is also conducted to the ventricle. This goes on for a variable time until a sinus stimulus finds the A-V system again refractory and is blocked. Because of the longer pause which follows, the conducted beats have longer refractory periods and a 2:1 block persists.

Discussion. The tracings described above show that we are dealing with ectopic stimulus formation and intraauricular, intraventricular and A-V conduction disturbances. No extrasystoles with fixed coupling were observed. This finding contradicts the common statement found in the literature that "extrasystoles" appear in veratrine intoxication since only those ectopic beats which are coupled to the preceding beat should be named extrasystoles(2). A study of the literature revealed that all tracings published as demonstrating ventricular extrasystoles actually show irregularly placed ectopic beats(2).

The absence of extrasystoles in these tracings is of interest because an attempt has been made in recent years to explain the appearance of extrasystoles early in diastole by the presence of a supernormal phase of excitability. The supernormal phase of excitability is closely connected with the negative after-potential. Since veratrine is known to increase the negative after-potential much more than any known substance extrasystoles would be expected to appear in these experiments. However, large negative after-potentials with very limited supernormality have been found (4), and it is certain that repetitive firing may

exist without a supernormal period and with a depressed excitability(5,6). The objection may be raised that in our experiments the method of anesthesia or the rate of respiration with a resulting alkalosis are responsible for the absence of extrasystoles. Study of the literature revealed, however, that other authors performing their experiments with a different technic and with other veratrine preparations also did not reproduce extrasystoles although they claimed to have done so(2). Injection of 6-8 cc of 0.05 N hydrochloric acid did not change the existing rhythms.

Conclusions. In the experiments reported above the intravenous administration of veratrine alkaloids did not lead to the appearance of extrasystoles with fixed coupling. A review of the literature on this subject did also not reveal this arrhythmia following veratrine. Only irregularly placed ectopic beats, ectopic tachycardias, intra-auricular, intraventricular and A-V conduction disturbances and finally ventricular fibrillation appear. The phenomenon of deblocking of a 2:1 A-V block by an extrasystole was described in the mammalian heart.

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Effect of Coconut Milk Fractions on Growth of Carrot Tissue.   (19994)

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(Introduced by J. J. Oleson)

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Since the discovery by van Overbeek *et al.* (1) of the growth promoting action of coconut milk for immature Detura embryos this material has been shown to enhance the growth *in vitro* of several plant tissues (2-6). In the present study the effect of coconut milk on the growth *in vitro* of a clone of carrot tissue was investigated and evidence was obtained that at least two fractions of the milk were concerned in the growth of this tissue.

Methods and results. Carrot tissue isolated from a single fragment of the phloem region of one carrot (var. Chantenay) was cultured on a medium containing 2% sucrose, White's mineral solution and 1% agar, to which the solid material from lyophilized coconut milk was added to give a concentration of 7 mg/ml. On this medium, the tissue was grown in darkness at a temperature of 25°C, with transfers every 2 weeks. This tissue was designated Clone II and, being genetically homogeneous, was used in all further experiments. This tissue has been grown in this way for over a year, showing a constant rate of increase of about 10 times the original dry weight during the 2 weeks period. *Coconut milk* pooled from batches of mature coconuts was stirred with Nuchar (C-1000-N)—equal to 2% of the solids of the milk—for 30 minutes. After filtration, the Nuchar cake was eluted 3 times with a chloroform-methanol (5:2) mixture. The combined eluates were evaporated to dryness *in vacuo* and the residue lyophilized. On an equivalent volume basis, the eluate averaged 0.2 mg/ml of solids as compared to 50 mg/ml of solids for the original milk. The original coconut milk and the filtrate and eluate fractions were incorporated in sucrose mineral agar and sterilized by autoclaving (15 lb for 15 min.). After being distributed to 18 x 150 mm pyrex tubes in 10 ml portions, a fragment of Clone II carrot tissue was placed on the surface of the agar in each tube. At the end of a 14 days incubation period in the dark at 25°C, the fragments were removed

TABLE I. Average Fresh Weight of Clone II Carrot Tissue after 14 Days Growth on Media Containing Coconut Milk and Fractions. All concentrations equivalent by volume to that of coconut milk (CM). Average of mean values from 9 assays.

Conc. of CM, mg/ml	Wt of carrot tissue (mg)			
	CM	Nuchar filtrate	Nuchar eluate	Eluate-filtrate
10	323	85	70	—
7	334	84	73	277
5	293	82	66	—
2	136	64	67	—
0	54	—	—	—

TABLE II. Average Growth in 14 Days of Carrot Tissue (Clone II) in the Presence of Filtrate and Eluate Factors from Coconut Milk. (Wet weights in mg.)

Filtrate conc., mg/ml	Eluate conc., mg/ml				
	.5	.1	.05	.01	0
7	414	341	205	168	89
1	206	191	147	123	70
.4	94	78	66	69	66
.0	74	74	56	58	54
.0	74	65	57	57	58

and their wet and dry weights determined. Five replicates for each concentration of fractions tested were used. As dry weights were proportional to wet weights, only wet weights are presented here. Nine separate batches of coconuts were treated in this way and assayed separately. The average weights from the 9 mean values obtained from a total of 1530 cultures are shown in second column of Table I. Agreement of the individual assays was fairly close, the standard error of the means being ± 10%. The samples assayed were original coconut milk, Nuchar filtrate, chloroform-methanol eluate and combinations of filtrate and eluate; concentrations of eluate and filtrate being equivalent by volume to that of the original coconut milk.

Results. As shown in Table I indicate that the clone of carrot tissue required two factors or groups of factors in coconut milk for its optimal growth response. The Nuchar eluate

TABLE III. Average Growth in 14 Days of Carrot Tissue (Clone II) in the Presence of the CM Filtrate Factor and Indole-3-Acetic Acid. (Wet weights, mg.)

Filtrate conc., mg/ml	IAA conc., mg/ml				
	.1	.01	.001	.0001	0
7	92	219	222	180	122
0	161	136	104	113	71

contains one group of the factors and the filtrate contains the other. Neither fraction alone was able to give the optimum response of the whole coconut milk. Table II shows that over the range of concentrations tested, the maximum response occurred when the filtrate was combined with the eluate in a ratio of 7.0 mg/ml filtrate to 0.5 mg/ml of eluate.

Since Caplin and Steward(5) reported that coconut milk may contain free indoleacetic acid (IAA), which might contribute to the activity of the eluate fraction, this possibility was investigated. The eluate was extracted 3 times with ether at pH 2.8. The ether extract gave no growth promoting response when combined with the filtrate. Moreover, the extract gave a negative response to the Salkowski test for IAA. Although the eluate fraction did not contain free IAA a comparative study was made to learn whether IAA could replace the eluate fraction. Addition of IAA to agar containing the filtrate factor resulted in an enhancement of growth, the maximum effects being at a concentration of 0.001 mg/ml (Table III). IAA without the filtrate factor enhanced the growth of the tissue to a greater extent than did the eluate fraction alone (Table I). The maximum growth enhancement of the carrot tissue by IAA alone was 2-fold, by IAA and filtrate fraction 3-fold, and 7-fold by the coconut milk eluate and filtrate combination. These results indicate that the activity of the eluate fraction is not due to IAA. Four other clones of carrot tissue were found to respond to the 2 fractions in a manner similar to Clone II. Shanz and Steward(7) have reported recently that 3 crystalline components from coconut milk singly will enhance the growth of carrot tissue only when supplemented with casein hydrolysate. It is possible that the casein hydrolysate exerts an effect similar to that of the coconut milk filtrate.

Summary. Addition of coconut milk (7 mg/ml) to a medium containing 2% sucrose, 1% agar and White's mineral solution supported the growth in darkness of Clone II carrot tissue for over a year through more than 100 transfers. Adsorption of coconut milk on Nuchar followed by elution with chloroform-methanol (5:2) gave two fractions, neither of which would support the growth of this tissue alone, but which, on recombination, had the same activity as the original coconut milk. The effect of the eluate factor could be partially reproduced by indole acetic acid. The filtrate factor could not be replaced by any of the chemically defined substances tested.

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Effect of Fasting on Blood Sugars in Hereditary Hypopituitary Dwarf Mice.* (19995)

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Snell(1) described, in a strain of mice, an hereditary dwarfism which behaved as a true Mendelian recessive. Since then many investigations have been carried out on dwarf mice which have shown interesting morphological and metabolic anomalies. Snell(1) described their thyroid deficiency and obtained restorative effects with a thyroid-stimulating extract, and Smith and MacDowell(2) showed that a hypoplastic condition existed in the acidophilic cells of the anterior pituitary lobe and in the sex glands, the skeleton, and the adrenal cortex. By transplantation of the anterior pituitary lobe tissue from rats to dwarf mice, normal growth could be achieved and other underdeveloped structures were restored to normal. Therefore, they concluded that this dwarfism was the result of an hereditary anterior pituitary deficiency. Kemp(3,4) showed that normal growth could be restored by replacement therapy with anterior pituitary extract and with growth hormone. MacDowell, Laanes, and Smith(5); Dawson(6); Osborn (7); Marshak(8); Boettiger and Osborn(9); Rouse and Osborn(10); Schonholz and Osborn(11); Haack(12); Mirand and Osborn (13); and other investigators have all reported observations related to the endocrine or morphological abnormalities of the hypopituitary dwarf mouse.

Since various endocrine glands and their extracts have been found to have an influence on metabolic processes, it was felt that the hereditary hypopituitary dwarf might serve as an excellent animal in which to study blood glucose values associated with fasting.

Materials and methods. The animals used

in this study were hereditary hypopituitary dwarf mice of the Bar Harbor strain, and for controls normal mice of the same strain were utilized. All dwarfs were 9 to 12 months old, ranging in weight from 9 to 14 g. Normal mice, 35 to 42 days old, served as controls, since their weights at this time were similar to the dwarfs, moreover, at this age the normal mice are developmentally similar to the dwarfs. The animals, bedded with ground corn stalks in solid-bottomed cages, were maintained on water and Purina laboratory chow. The temperature of the animal room was about 75°F. Experiments designed to determine the effects of fasting and nonfasting upon the blood sugar level of normal and of dwarf mice were carried out. The fasting periods used ranged from 12 to 96 hours, as indicated in the accompanying tables. Blood samples for sugar determinations were drawn after 10 p.m. While being bled, the mice were held gently to minimize the possible effect of excitement upon the blood sugar level. Blood for all sugar analyses (modified Folin-Wu methods of Klendehoj-Hubbard) was obtained by using the following technic. The animal's tail, having been stroked and warmed to promote vascular dilation and blood flow, was nicked with a sharp razor. If cut properly to include a prominent subcutaneous blood vessel, large drops of blood quickly form but the tail will heal promptly. Fresh blood was quickly drawn in a Sahli pipette and any excess wiped off the stem. The blood was then chemically prepared and the colors compared in a Klett colorimeter. The blood sugar values obtained are consistent relative amounts rather than absolute ones.

Results. Blood sugar determinations on fed and fasted dwarf and normal mice. In Tables I and II are shown the average blood glucose values for fed dwarf mice with normal controls and for dwarfs and normals fasted 12 to 96 hours. In the fed state the average

TABLE I. Effect of Fasting on Blood Sugar of Normal and Dwarf Mice.

	Normal				Dwarf			
No. in series	Hr fasted	Blood sugar, mg %	S.E.	No. in series	Hr fasted	Blood sugar, mg %	S.E.	t-value
10	0	155	± 4.26	21	0	143	± 4.96	.18*
50	12	147	± 2.21	15	12	110	± 3.13	10
18	15	128	± 2.23	10	15	105	± 2.81	6.38
37	16	123	± 1.82	15	16	100	± 4.56	6.21
28	18	119	± 2.21	15	18	94	± 4.35	5.10
18	21	124	± 2.47	10	21	95	± 3.98	6.17
50	24	133	± 2.18	15	24	93	± 2.98	10.81
12	27	131	± 2.56	15	27	97	± 1.82	12.50
30	30	137	± 2.23	15	30	92	± 5.08	8.03
24	40	138	± 3.36	15	40	88	± 2.68	11.36
12	48	117	± 1.23	12	48	75	± 1.71	21
12	72	112	± 1.44	12	72	71	± 1.87	17.91
12	96	103	± 1.03	12	96	68	± 1.86	15.90

* Not significant; all other values are significant.

TABLE II. Sequential Blood Sugar Determinations of 10 Normal and 10 Dwarf Mice Subjected to Fasting.

	Normal		Dwarf		
Hr fasted	Blood sugar, mg %	S.E.	Blood sugar, mg %	S.E.	t-value
* 0	155	± 4.3	143	± 5	.18†
12	149	± 2.2	108	± 2.1	13.66
15	126	± 3.1	102	± 1.8	6.66
18	125	± 5.2	97	± 1.9	5.09
24	130	± 1.2	92	± 2.2	11.51
25	129	± 3.1	95	± 1.5	9.71
26	134	± 1.8	91	± 2	16.54
27	133	± 2.1	88	± 1.6	17.30
28	135	± 1.4	90	± 1.6	20.45
29	133	± 2.5	89	± 1.2	15.71
30	138	± 1.3	92	± 3.3	12.22
36	131	± 2.7	87	± 2.5	11.89
40	128	± 2.9	85	± 3.6	8.69

* Control mice.
† Not significant; all other values are significant.

blood sugar values for the dwarf mice (143 mg %) are slightly, but not significantly, lower than those of the normal mice (155 mg %). The blood sugar level of the fasting dwarf drops most precipitously around the 18th hour, followed by a more gradual but steady decline to an average value of 68 mg % after 96 hours of fasting. In contrast, normal mice exhibit the greatest blood sugar drop between the 12th and 18th hours of fasting, followed by some recovery between the 21st and 40th hours of fasting. Thereafter, a gradual second drop occurs, reaching a minimum of 103 mg % at the 96th hour. It is to be emphasized that, although prefasting blood sugar values are essentially alike for dwarfs and normal controls, the normal mice are able to maintain high levels rather tenaciously even during fasting, while dwarfs exhaust their blood sugars steadily and rapidly.

Table II presents the average blood sugar values from sequential blood sugar determinations on 10 normal and 10 dwarf mice subjected to fasting for 12 to 40 hours. Sequential determinations represent blood sugar analyses on a series of blood samples taken from the same mouse at intervals indicated during the fasting. These data are in close agreement with the results in Table I, despite the difference in sampling. It is clear, therefore, that dependable values may be obtained when multiple blood samples are taken from the same animal. These findings show the sensitiveness of the dwarf to fasting to a degree that the dwarf symptoms resemble the observations of others reported for hypophysectomized animals. Although in the dwarf a progressive decrease in the blood sugar level occurs, this decline is not associated with hypoglycemic convulsions and death, contrasted with hypophysectomized animals which may succumb if fasted for only 24 hours (14). In the fasting dwarf, activity is curtailed progressively until, by the end of the 96-hour fast, the dwarfs are either extremely lethargic or dead. In this study about one-third of the dwarfs expired by the 96th hour despite efforts to elevate the blood sugar by administering 5% glucose by stomach tube.

Starvation effects were not superficially discernible in normal mice at the end of the 96-hour fast.

Discussion. When hypophysectomized animals are fasted, they suffer unusually rapid losses of body carbohydrates. As seen in this study, the dwarf mice behave somewhat like hypophysectomized animals in that after a fast of 96 hours the blood sugar level of the dwarf is considerably lower than that of normal animals. It is generally agreed that the secretion of the adrenal cortical steroid hormones is largely controlled by anterior pituitary adrenocorticotrophin (ACTH). The dwarf, representing a hypopituitary state, is deficient in ACTH(12). It may be suggested, therefore, that the hypoplastic condition of the anterior pituitary and the adrenal cortex in the dwarf is associated with functional deficiencies of hormones which are normally concerned with the metabolism of carbohydrates. This imbalance in the adrenal-pituitary axis in the dwarf might best explain the severe hypoglycemia which develops under the stress of fasting.

However, in the light of the researches of Houssay and Anderson(15), and De Bodo *et al.*(16) relating the growth hormone to carbohydrate metabolism, it is not unlikely that the unique blood sugar pattern found in the fasting dwarf mouse may be partly explained by the animal's deficiency in growth hormone. Experiments designed to gain information on this problem are in progress.

Summary. Under the experimental conditions employed, findings may be summarized as follows: 1. Average blood sugar values for fed hereditary hypopituitary dwarfs are slightly, but not significantly, lower than those of fed normal mice. 2. The blood sugar level of fasting dwarfs drops precipitously to a minimum of 68 mg % after 96 hours. In contrast, normal mice exhibit a lesser drop, followed by some recovery which later gives way to a minimum average of 103 mg % after 96 hours of fasting. 3. These findings show that the sensitiveness of the dwarf to fasting is somewhat like that reported for hypophysectomized animals. This sensitivity herein reported, as judged by the severe hypoglycemia which develops under the stress of fasting, might best be attributed to the known imbalance in the adrenal-pituitary axis in the dwarf.

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Toxicity of Autoclaved Cystine for *Lactobacillus bifidus*.* (19996)

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In connection with studies on the ability of different sugars to support the growth of *Lactobacillus bifidus* and its unbranched mutants(1), it was observed that if any of the sugars was sterilized by filtration and added to autoclaved, sugar-free medium, there was usually a negligible amount of growth. Orla-Jensen(2) found that for growth of lactobacilli sugar must be autoclaved with the medium or with the nitrogen source (yeast extract). Sugar autoclaved separately and added to autoclaved medium did not support growth. The effect was attributed to the action of decomposition products of sugar such as methylglyoxal. Methyglyoxal itself was tested and found to be effective, but it also had to be autoclaved with the yeast extract. Smiley, Niven, and Sherman(3) working with *Streptococcus salivarius,* and later Rabinowitz and Snell(4) and Snell, Kitay and Hoff-Jorgensen(5), using *L. bulgaricus* observed a somewhat different reaction. They found that growth of the organisms was supported when the sugar was autoclaved separately under alkaline conditions and added to autoclaved medium. It was suggested that in the presence of such decomposition products of sugar as pyruvic acid, a favorable oxidation-reduction potential was developed in the medium. Pyruvic acid could, in fact, replace the autoclaved sugar. The present investigation describes studies carried out to determine what factors were involved in the requirement of *L. bifidus* for autoclaved sugar. The first experiments were directed toward identification of a possible growth stimulant, either a degradation product of sugar or some substance formed by reaction of sugar with amino acids, such as the compounds described by Maillard(6) and others. It was found that sugar autoclaved with cystine supported growth in medium autoclaved without sugar.

Subsequent tests indicated, however, that this effect did not depend on the formation of a growth stimulant during autoclaving since the same result could be obtained by using sugar and cystine sterilized by seitz-filtration, or indeed by sterilizing the whole medium by seitz-filtration instead of by autoclaving. The medium contained a high level of cystine, 2 mg per tube, since it has been shown that there may be extensive destruction of sugar during autoclaving(7,8). It was established that failure of *L. bifidus* to grow in medium autoclaved in the absence of sugar was a result of the formation in medium so treated of some toxic decomposition product of cystine. Sugar autoclaved with the medium prevented the formation of such products by its reducing power. The toxicity could be overcome by adding to the autoclaved medium certain reducing substances, in particular those containing sulfhydril groups.

Experimental. Studies were made on *L. bifidus* and the unbranched mutant, Strain A, which have been described(9). Since the results with the two types of organism were identical, data will be presented only on the bifid strain. The medium was the modification of the Teply-Elvehjem medium used by Norris, *et al.*(9). Double strength medium (without sugar) was prepared in advance and sterilized in 2 liter flasks by autoclaving for 10 minutes at 15 lb pressure. At the time of the experiment, 5 ml of basal medium was transferred to each culture tube, any desired supplements were added, and the tubes were autoclaved for 10 minutes. Ascorbic acid (1 ml of a 1% solution) was always seitz-filtered and added aseptically to the autoclaved medium. Where noted, sugar and other supplements were either autoclaved separately from the medium or seitz-filtered and added aseptically. Solutions were prepared so that the amount of supplement for each test was contained in 1 to 3 ml. In all cases the

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TABLE I. Production and Prevention of Cystine Toxicity in Autoclaved Medium.

Treatment of sugar-free medium	Treatment of lactose	Supplements, mg		Acid production, ml .1 N in 40 hr
		Exp. 1—Regular medium		
Autoclaved	Autoclaved with medium			*21.4 ± .4
"	" separately, pH 10			.1
"	Seitz-filtered			.2
"	"	Pyruvic acid, 10		.5
"	"	Cystine, 6		22.4
"	"	Cysteine · HCl, 6		22.7
"	"	Thiomalic acid		17
"	"	Thioglycollic acid	Seitz-filtered	15.5
"	"	Ascorbic acid,‡ 15		0
"	"	" " ‡ 40		0
"	"	Reductone, 50		12.9
"	"	Ascorbic acid,‡ 40 + reductone, 50		15
Seitz-filtered	"			21.7
		Exp. 2—Medium with no added cystine		
Autoclaved	Autoclaved with medium			8 †
"	Seitz-filtered			14.2†
"	Autoclaved with medium	Cystine, 2	Autoclaved with med.	22
"	Seitz-filtered	" 2	" " "	7.7
"	Autoclaved with medium	Cysteine · HCl, 2	" " "	21.7
"	Seitz-filtered	" " 2	" " "	23.4
"	"	Cystine, 2	separately, pH 7	.4
"	"	" 2	" pH 1.5	.4
"	"	Cysteine · HCl, 2	" pH 1.5	21.6
"	"	" " 2	Seitz-filtered	23.8
		Exp. 3—Medium with no ascorbic acid		
Autoclaved	Seitz-filtered	Cystine, 6	Seitz-filtered	11.6
"	"	Cysteine · HCl, 6	"	20.5

* Data in Exp. 1 have been taken from four assays. Since the control titration was almost the same in the 4 tests, individual experiments have not been tabulated separately.

† The difference between these samples was even more marked at 16 hr when the titrations were 1.8 ml and 7 ml.

‡ In addition to the 10 mg of ascorbic acid routinely present in the medium.

final volume in each tube was 10 ml. The concentration of sugar used was 350 mg per tube. In the experiments reported lactose was used. Similar results were obtained when glucose or galactose was substituted for lactose.

If for *L. bifidus* as for *L. salivarius* and *L. bulgaricus*(3-5) the favorable effect of autoclaving sugar depended on the formation of some breakdown product, addition of autoclaved sugar to medium sterilized without sugar should stimulate growth. However, sugar autoclaved separately from the medium under alkaline conditions allowed only slight growth. Neither did addition of pyruvic acid (10 mg per tube) stimulate growth (Table I, Exp. 1). To test the possibility of the formation of a growth stimulant by a Maillard reaction, lactose was autoclaved with a mixture of alanine, cystine, and tryptophane. These 3 amino acids were always present in the medium as supplements to the casein digest used as the source of nitrogen; the amount added with the lactose doubled the usual concentration. When the autoclaved lactose-amino acid mixture was added to medium autoclaved without sugar, growth was as good as when the lactose was autoclaved with the medium. When each amino acid was tested individually by autoclaving it with lactose, it was found that only the combination of cystine and lactose supported growth.

These findings appeared to be in accord with the assumption that interaction of lactose with cystine during autoclaving might lead to formation of a specific growth stimulant. However, this hypothesis became untenable when it was found that the mixture of cystine and

lactose was as effective when it was sterilized by seitz-filtration as when it was autoclaved (Table I, Exp. 1). That the value of auto-claving cystine with sugar did not depend on the formation of a growth stimulant was veri-fied by sterilizing the whole medium by filtra-tion. Unautoclaved medium promoted growth as effectively as did autoclaved.

In another series of experiments (Table I, Exp. 2) the basal medium contained no supple-ment of cystine, so that the only source of this amino acid was the casein digest which fur-nished about 0.2 mg per tube. This low-cystine medium also was autoclaved with and without lactose. When the lactose was added before autoclaving growth of the organism was very poor. However, when the lactose was added aseptically to medium which had been autoclaved, acid production approached maximum rate and amount. These results were the opposite of those obtained with medium of high cystine content: in high-cystine medium autoclaving with sugar was essential, growth in medium to which the sugar was added after autoclaving being small or completely inhibited, whereas in low-cystine medium autoclaving in the absence of sugar allowed good growth while addition of sugar before autoclaving reduced acid pro-duction to about one-third the usual level.

It has been reported on the basis of both chemical(10) and microbiological(7,8) studies that the presence of reducing sugar increases the rate of destruction of cystine and cysteine during autoclaving. It is probable that in the low-cystine medium in the presence of sugar so much cystine was destroyed during autoclaving that full growth of the organism was impossible, while in the absence of sugar or in medium supplemented with cystine, the cystine level was not reduced below the amount required for good growth. That there was no growth in high-cystine medium auto-claved in the absence of sugar in contrast to the excellent growth in low-cystine medium might be explained by the formation of some inhibitory substance from cystine, which was present in effective amount only when the level of cystine in the medium was high. Evi-dence for the toxic origin of growth inhibition in the high-cystine medium autoclaved without

sugar was furnished by experiments in which cystine in aqueous solution (2 mg in 3 ml water), autoclaved for one-half hour, was found to inhibit the growth of *L. bifidus* when it was added to autoclaved complete medium at the usual level of 2 mg per tube. Cysteine, on the other hand, was not toxic whether autoclaved with the medium or separ-ately (Table I, Exp. 2).

It has been mentioned that cystine or cysteine added aseptically with lactose to high-cystine medium autoclaved without sugar were effective in overcoming the inhibitory effect of the autoclaved cystine. Cystine was as effective as cysteine only when the medium contained ascorbic acid. In medium from which the ascorbic acid was omitted the rate of acid production was much slower with cystine than with cysteine (Table I, Exp. 3). Thioglycollic and thiomalic acids could be substituted for cystine or cysteine, but were somewhat less effective. Ascorbic acid up to a level of 50 mg was ineffective in counteracting the effect of autoclaved cystine. In contrast, reductone† at the 50 mg level allowed con-siderable growth, although it was much less effective than the sulfhydril compounds.

The protective effect of autoclaving cystine in the presence of lactose was probably due to the reducing properties of lactose. A non-reducing sugar would not be expected to prevent the development of toxicity in auto-claved cystine. This would explain the fact that under the usual experimental conditions sucrose did not support the growth of *L. bi-fidus*(1). There was growth on sucrose when it was used in unautoclaved medium or in medium supplemented, after autoclaving, with unautoclaved cysteine, although even under these conditions neither the rate nor the total amount of growth was as great as with lactose.

Discussion. The toxic substance formed from cystine was presumably an oxidation product since autoclaving the medium in the presence of reducing sugar prevented its ap-pearance and the toxicity was reversed by the addition of cysteine or related sulfhydril compounds or of reductone (ene-diol form of

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† Kindly furnished by Professor R. Kuhn, Heidel-berg.

hydroxymethylglyoxal). It is of interest that reductone was active whereas ascorbic acid was ineffective since both owe their reducing activity to an ene-diol structure.

Schuhardt *et al.*(11) and Rode *et al.*(12) found that cystine when autoclaved in neutral solution (but not when autoclaved at pH 1.5) was toxic for brucellae, and explained the toxicity of some lots of tryptose by the formation of oxidation products of cystine on standing. Under the conditions of our experiments cystine was toxic after autoclaving in acid as well as in neutral solution. Recently Schuhardt *et al.*(13,14) have identified the substance toxic to brucellae as elemental sulfur. Following this report the toxicity of colloidal sulfur‡ for *L. bifidus* was tested. Growth of the organism was inhibited by sulfur added to the autoclaved medium but the amount required for complete inhibition was large, about 10 γ per ml, whereas the growth of brucellae had been inhibited by 0.1 to 0.2 γ of sulfur per ml. The toxic effect of the sulfur could be reversed by autoclaving it with the complete sugar-containing medium or by addition of cysteine with the sulfur.

These experiments emphasize the importance and variety of interactions among the components of the medium, particularly under the severe conditions imposed by autoclaving. With a high level of cystine in the medium autoclaving with sugar was beneficial, with a low level of cystine, detrimental. Low concentrations of sugar were not tested but would

‡ The method by which the colloidal sulfur used in his experiments was prepared was kindly furnished to the authors by Dr. V. T. Schuhardt.

surely modify the picture. An apparent complete failure of sucrose to support growth in the usual medium was a reflection of the fact that it is not a reducing sugar.

Summary. Autoclaved cystine is toxic to *L. bifidus*. Autoclaving in the presence of reducing sugar prevents development of the toxic product. The toxicity may be counteracted by addition, after autoclaving, of cysteine, thioglycollic or thiomalic acids, reductone, or cystine itself with ascorbic acid, but not by addition of ascorbic acid alone.

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Mono- and Disaccharides in Growth of *Lactobacillus bifidus* and Its Mutants. (19997)

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Wright(1) described a strain of *Streptococcus thermophilus* which grew more readily with lactose or sucrose than with any other

sugar. Since that time, several workers have discussed organisms which utilize disaccharides more readily than their constituent

monosaccharides. Hestrin(2) has reviewed the literature and discussed possible mechanisms for this direct utilization of disaccharides.

Recent studies in this laboratory indicate that *Lactobacillus bifidus* is a member of the group of organisms which utilize disaccharides preferentially. Norris, Flanders, Tomarelli, and György(3) have reported the isolation and maintenance of a number of strains of a microorganism with the morphologic and metabolic characteristics of *L. bifidus* which was first described by Tissier in 1899(4). These authors discussed also unbranched strains of lactobacilli which appeared to be derived by mutation from the branched strains and resembled certain standard strains of *L. acidophilus*, in particular, the strains of Weiss and Rettger which were originally designated *L. bifidus*. In the present communication a difference between the bifid and unbranched strains in carbohydrate utilization is reported. Whereas *L. bifidus* fermented lactose or maltose more rapidly than glucose or galactose, the unbranched strains, as well as various standard strains of lactobacilli utilized monosaccharides as well as or better than the disaccharides.

Experimental. The organisms studied included 5 strains of *L. bifidus* isolated in this laboratory and maintained in subculture over a period of 2 years, 2 unbranched strains, "A" and "I" which may be considered as mutants of the bifid strains, and 5 cultures of *L. acidophilus* obtained from the American Type Culture Collection: No. 9857, Döderlein's bacillus; Nos. 4355 and 4357, a murine and a human strain isolated by Kulp; and Nos. 4962 and 4963, strains which were originally classified by Rettger as *L. bifidus* and which are very similar to strain "A"(3). All of these strains were carried on a modification of the Teply and Elvehjem medium as described by Norris *et al.*(3). The inoculum, medium, and general procedures used in these studies have been discussed by Tomarelli *et al.*(5). The minimal medium satisfactory for growth of the bifid strain was modified to provide optimal conditions for the various organisms studied. One unbranched strain, "I", required desoxyriboside for growth, and

strains "A" and "I" as well as several of the ATCC strains required unsaturated fatty acid. The first of these needs was met by use of an enzymatic digest of casein as the source of nitrogen. The pancreatin present in this preparation contains desoxyriboside. Unsaturated fatty acid was supplied as Tween 80, a polyoxyethylene derivative of sorbitan monooleate which, unlike free unsaturated fatty acids, was not toxic to the bifid organisms (6). When sodium citrate was used as the buffer in the medium 100 mg of dicalcium phosphate, 10 mg of manganese sulfate and 5 mg of magnesium sulfate were added to each tube to compensate for the binding of bivalent ions by citrate. Only the calcium salt was necessary for most of the unbranched strains, but the branched strain required the additional supplement of manganese and magnesium ions for regular and consistent growth. The concentration of sugar in the medium was 3.5%. Growth of the organisms was measured by the amount of acid produced.

All of the strains of *L. bifidus* studied utilized lactose more readily than glucose in marked contrast to the unbranched variants derived from them. In Table I are given data of an experiment in which the acid production of a bifid strain is compared with that of Strain A, a straight mutant, when they were grown in media containing lactose or one or both of its constituent monosaccharides. In the medium buffered with sodium acetate there was good growth of both strains after 64 hours, regardless of the type of sugar present. However, in 20 hours the bifid strain grew well only with lactose, the straight strain only with glucose. When the sodium acetate of the medium was replaced by sodium citrate, a buffer which is less satisfactory for the growth of these organisms(6), the difference between the 2 strains was more marked. In this medium the bifid strain grew excellently on lactose, but very poorly on glucose, even after 64 hours. The straight strain, on the contrary, grew negligibly on lactose in 64 hours, while with glucose, it produced half the maximum amount of acid in 20 hours. Growth with galactose was intermediate between that with glucose and lactose for both strains. An equimolecular mixture of glucose

TABLE I. Utilization of Sugars by *L. bifidus* and an Unbranched Variant.

Buffer of medium	Sugar (350 mg/tube)	Acid production (ml .1 N per 10 ml culture)					
		Bifid strain			Unbranched strain (A)		
		20 hr	40 hr	64 hr	20 hr	40 hr	64 hr
Acetate	L*	9	23.6	23.7	3.6	10.1	13.1
	Gl	1.6	14.6	17.1	11	16.8	17.1
	Ga	1.1	14.7	19.5	3.5	8.2	9.3
	Gl + Ga†	1.9	15.9	19.3	10.5	12.5	12.4
Citrate	L	.8	19.5	23.6	0	.7	1.1
	Gl	0	4.1	6.2	7	12.4	10.6
	Ga	0	7.7	16.5	1.1	2.8	7.8
	Gl + Ga†	0	10.3	13.1	6.9	8.9	8.7
Acetate	L	3.2	17.1	24.1			
	M	1.8	14.2	18.2			
	S	0	0	0			
Acetate	L added aseptically	4.3	20.4	24	Cysteine, 6 mg, added asep.		
	M " "	3	14.1	16.9	" " " "		
	S " "	0	6.6	18.3	" " " "		

* L = Lactose, Gl = Glucose, Ga = Galactose, M = Maltose, S = Sucrose.
† 175 mg of each.

and galactose was not equivalent to lactose in supporting growth of the bifid organism.

All of the unbranched strains of lactobacilli studied resembled strain A in their utilization of glucose and lactose. None of them grew better on lactose than on glucose. Two strains used glucose and lactose at the same rate; the other strains metabolized glucose faster than lactose. This advantage of glucose persisted even though the strains had been carried for at least several months in medium containing lactose as the only sugar.

In other experiments the relative growth promoting activity for the bifid strain of the disaccharides, sucrose, lactose and maltose was compared (Table I). Maltose was almost as effective as lactose, but under the usual experimental conditions sucrose was inactive. It was found, however(7), that medium which did not contain reducing sugar acquired toxic properties during autoclaving. This difficulty was circumvented by sterilizing the sugar together with cysteine, by seitz-filtration and adding the mixture to medium autoclaved without sugar. When this procedure was followed, there was some growth with sucrose, but the rate was much slower than with lactose or maltose.

Since glucose was so poorly utilized by the bifid strain, cultures grown on lactose were tested for the presence of glucose by the glucose oxidase method of Keilin and Hartree

(8). It was found that glucose accumulated slowly during the incubation period being 0.3% after 24 hours, 0.8% after 40 hours, and 1.0% after 64 hours. This result offered the possibility that the organism was making effective use only of the galactose portion of the molecule. However, assay for galactose and glucose by the procedure of Sprague and Bellamy(9) using *Streptococcus mastiditis* 68 showed that glucose and galactose were present in the culture in approximately equal amounts. An unbranched strain tested at intervals from 8 to 64 hours contained at no time sufficient glucose to be detected by the glucose oxidase method.

Discussion. The several strains of *Lactobacillus bifidus* studied were distinct from their unbranched variants and from the strains of *L. acidophilus* tested in that they metabolized lactose more readily than glucose. The straight rods utilized glucose at least as well as, and usually better than lactose even though they were routinely carried in a medium containing lactose. Adam(10) reported that lactose, maltose, and sucrose, in decreasing order of efficiency were superior to monosaccharides for the growth of *L. bifidus*. He based his conclusions on the number and morphology of organisms observed in gram-stained preparations. Orla-Jensen, Orla-Jensen, and Winther(11), and Olsen(12), who studied the fermentation of a large number of carbohy-

drates by *L. bifidus* did not observe significant differences among the sugars we have studied. However, they measured acid production after a 2-week incubation period so that differences in the rate of fermentation such as we observed in 20 to 64 hours were not apparent.

Organisms which grow preferentially on disaccharides vary in the specificity of their requirements. A strain of *L. bulgaricus* studied by Snell, Kitay, and Hoff-Jorgensen(13) used only lactose of the disaccharides but grew on a number of B-galactosides. The strain of *Streptococcus thermophilus* discussed by Wright(1) grew best on lactose and sucrose. *L. bifidus* used sucrose to only a limited extent but metabolized both maltose and lactose readily.

It has been noted by a number of workers that even though an organism used a disaccharide much better than one or both of the constituent monosaccharides there was no accumulation of monosaccharides during the growth period(13-15). Doudoroff *et al.*(15) using dried cell preparations of a mutant of *Escherichia coli* which utilized maltose did observe accumulation of glucose and his suggested mechanism accounts for its formation. However, glucose was never found in more than trace amounts when intact cells were used. *L. bifidus* on the contrary does show accumulation of both glucose and galactose indicating that it does contain an active lactase which converts a considerable portion of the lactose present to glucose and galactose but lacks the enzyme system to make effective use of these sugars.

Summary. 1. *Lactobacillus bifidus* utilizes the disaccharides lactose and maltose, but not sucrose, more readily than the monosaccharides, glucose and galactose. The reverse is true of straight rod variants of *L. bifidus*. Several strains of *L. acidophilus* utilize glucose or lactose equally well or use glucose more readily. 2. During growth of *L. bifidus* on lactose, glucose and galactose accumulated in the medium. The straight rod mutant of *L. bifidus,* on the other hand, utilizes lactose completely.

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Studies on Phenylpyruvic Oligophrenia. Phenylpyruvic Acid Content of Blood.   (19998)

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Phenylpyruvic oligophrenia (phenylketonuria) is a form of mental deficiency characterized by the urinary excretion of phenylpyruvic, phenyllactic, phenylacetic acids and phenylalanine(1). Although abnormally high amounts of phenylalanine have been observed

in the blood of these patients(2,3), the presence in the blood of the deaminated phenyl-acids has never been reported. The purpose of this note is to report data on the blood content of phenylpyruvic acid and to comment briefly on their significance.

Materials and methods. The subjects were 39 phenylketonuric patients ranging in chronological age from 4 to 68 years and in intelligence quotient from 5 to 51. Blood for examination was obtained after 10-12 hours fasting and, during feeding experiments, one, 2 and 6 hours after the ingestion of the compound fed. For the quantitative determination of phenylpyruvic acid, the color reaction with ferric chloride was utilized which was first described by Erlenmeyer(4) and previously applied to the estimation of the acid in the urine(5-7). Some 30 ml of heparinized blood was immediately centrifuged and the plasma separated. Exactly 50 ml (corresponding to 10 ml of plasma) of a 1 to 5 sulfuric acid-tungstate filtrate was prepared in the routine manner, acidified to Congo red and extracted 3 times with 40 ml of ether. The combined ether was dried with anhydrous sodium sulfate and evaporated in vacuum. The residue was dissolved in 5 ml of glycine-sodium chloride-hydrochloric acid buffer of pH 2.2, filtered and to 4 ml of the clear filtrate, 0.5 ml of a freshly prepared 1% aqueous solution of ferric chloride was added. The green color was read in a Coleman junior spectrophotometer at 600 wavelength exactly 2 minutes after mixing. A standard curve was prepared using various amounts (20 to 300 μg) of twice recrystallized phenylpyruvic acid dissolved in 50 ml of water and treated exactly as the blood filtrate. In all readings the reagent blank was used as reference. Under these experimental conditions, samples of blood from 20 non-phenylketonuric healthy individuals of various chronological ages and intelligence quotients, gave values corresponding to .012-.016 mg/100 ml of plasma. Since no evidence could be obtained indicating that this figure represents phenylpyruvic acid normally present in the blood, the value .015 mg was arbitrarily subtracted from all readings. The chemicals used were commercial preparations with the exception of phenylpyruvic and

TABLE I. Phenylpyruvic Acid Content of Blood.

Patient	C.A.	I.Q.	Wt deviation, kg	Phenylpyruvic acid, mg %
1. A.	36	7	+ 9	.53
2. B.	68	23	— 5	.40
3. B.J.	45	47	+18	.73
4. C.	8	32	— 2	.63
5. C.J.	6	26	+ 3	.83
6. C.B.	31	37	— 6	.73
7. C.W.	30	7	—12	.66
8. D.	28	30	+ 3	.58
9. D.Al.	23	9	— 7	.36
10. Do.	41	17	— 2	.44
11. D.M.	38	20	—10	.83
12. D.E.	25	31	— 2	.40
13. F.	32	5	+ 7	.83
14. F.A.	4	36	+ 4	.90
15. Gi.	28	13	—10	.31
16. G.	12	36	+ 1	.66
17. G.S.	9	44	+ 2	.70
18. G.E.	33	5	+15	.49
19. H.	32	11	+ 3	.70
20. K.	9	24	— 6	.36
21. K.A.	33	43	— 5	.39
22. K.P.	5	23	— 4	.42
23. K.H.	11	9	—13	.66
24. K.J.	10	10	— 6	.60
25. K.R.	26	6	+12	.44
26. K.W.	37	26	+14	.80
27. L.	52	51	— 2	.53
28. M.	18	21	—10	.63
29. M.M.	35	11	+ 8	.52
30. M.R.	24	31	+ 2	.52
31. N.	27	7	— 6	.60
32. P.	17	6	—14	.36
33. P.J.	10	8	—16	.31
34. Q.	33	13	— 9	.31
35. R.	32	47	+16	1.78
36. S.	4	32	+ 2	.66
37. S.N.	28	20	— 3	.42
38. S.E.	31	32	— 2	.66
39. W.W.	53	42	— 6	.38

Mean .714 S.D. .248 ± .018

phenyllactic acids which were prepared in the laboratory following standard procedures.

Results. In Table I, the results of the determination of phenylpyruvic acid in 39 patients are presented. The patients are in alphabetical order; C.A. is chronological age; I.Q. the intelligence quotient; deviation from normal weight was calculated from standard tables taking into consideration age, weight and height of the subject; phenylpyruvic acid values are expressed in mg per 100 ml of plasma. It may be seen that the keto-acid is present in the blood of every patient. The coefficient of correlation for degree of mental deficiency and amount of phenylpyruvic acid present in the blood is +.434 ± .084. If
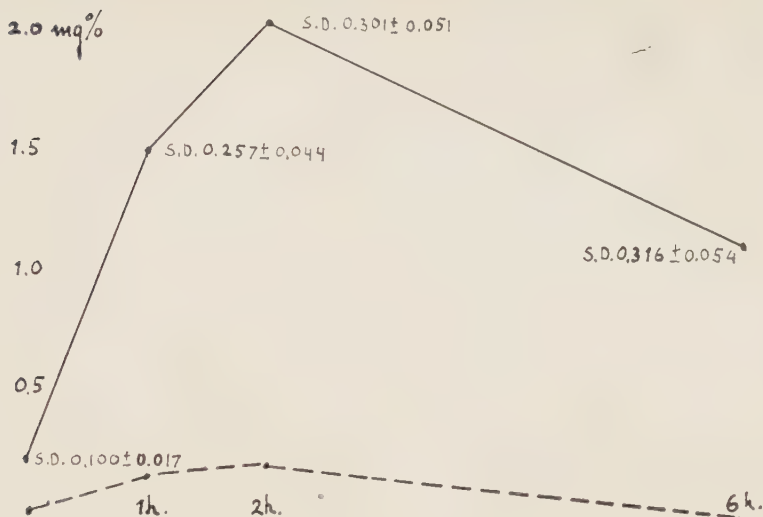
FIG. 1. Phenylketonemic curve following ingestion of DL phenylalanine (10 g) in 8 patients (upper curve, mean values) and 2 controls (lower curve).
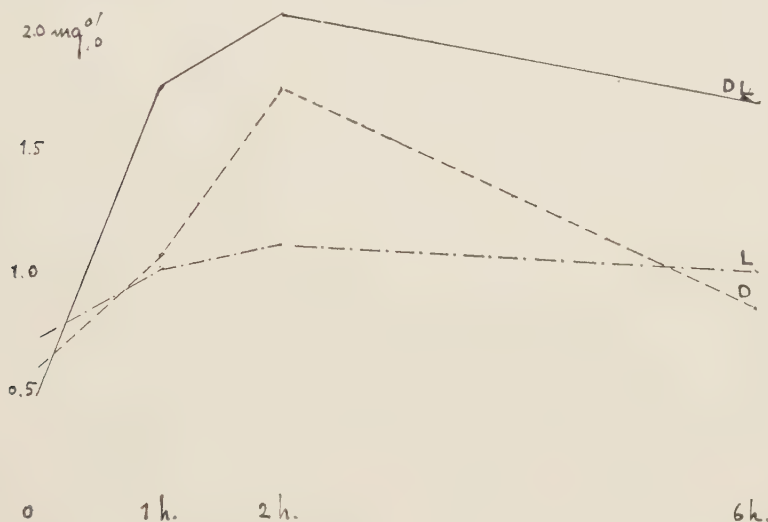
FIG. 2. Phenylketonemic curve following ingestion of DL (10 g), D (5 g) and L phenylalanine (5 g).

phenylketonemia is correlated with nutritional state as measured by the deviation from normal weight, the coefficient is $+.470 \pm .083$.

Eight adult patients whose weight averaged normal value were fed 10 g DL-phenylalanine. In Fig. 1 the phenylketonemic curve is shown. It represents the mean value at the first, second and sixth hour. It may be seen that the increase averaged about 4 times the fasting value. The peak of the increase was at about the second hour but elevated values were still apparent 6 hours after the feeding. In 2 non-phenylketonuric healthy adults of normal weight, a slight increase (.4 mg/100 ml) was observed under the same experimental conditions. During the 6 hours of this experiment, 3 phenylketonuric patients showed a urinary excretion of phenylpyruvic acid approximating 1200 mg in excess of the amount excreted during the same period of time the day prior to the feeding. The 2 normal controls excreted 553 and 675 mg, respectively.
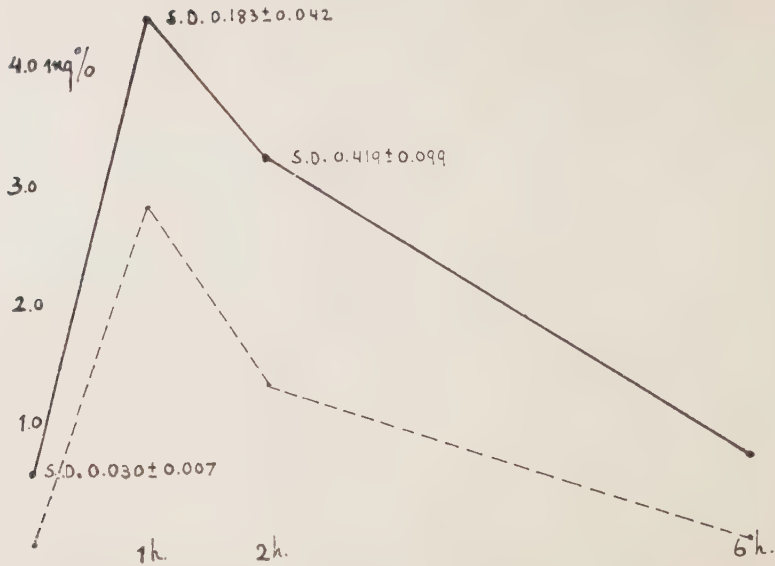
FIG. 3. Phenylketonemic curve following ingestion of phenylpyruvic acid (4.5 g) in 5 patients (upper curve, mean values) and 2 controls (lower curve).

In Fig. 2, the results of feeding the D and L phenylalanine to one patient are seen. It is apparent that the increase of phenylpyruvic acid in the blood is due mostly to the D form although a significant increase is shown also following the feeding of the L form. In this experiment, 10 g of DL and 5 g of L and D forms, respectively, were fed. In a control subject the L form (5 g) produced no appreciable effect while a small increase was observed following feeding of the D form (5 g). This control subject excreted 410 mg keto-acid following D phenylalanine and none following the L form.

In Fig. 3, the results of feeding 4.5 g phenylpyruvic acid to 5 phenylketonurics are shown. In all patients there was a sharp increase of phenylketonemia averaging 7 times the fasting value at the first hour. In 2 controls the type of curve was similar to that of phenylketonurics although the peak of the increase was some 25% lower. It is pertinent to note that in a non-phenylketonuric subject suffering from acute infectious hepatitis, the phenylketonemic curve following the ingestion of 4.5 g phenylpyruvic acid reached 4.6 mg/100 ml after one hour and 2.75 mg after the second hour, the curve being similar to that shown by phenylketonurics.

In 2 patients, 2 g per day of Benemid (p-di-n-propylsulfamylbenzoic acid) were administered for 3 days prior to feeding phenylalanine and phenylpyruvic acid. The resulting phenylketonemic values are seen in Table II. It is apparent that Benemid had the effect of raising significantly the phenylketonemic curve. In phenylketonurics kept on nitrogen constant diet, the ingestion of Benemid resulted in some 50% decrease of urinary excretion of phenylpyruvic acid.

Following the feeding of 5 g of the calcium salt of phenyllactic acid to one patient, there was an increase of phenylketonemia. The following values were observed: at one hour 2.60 mg/100 ml of plasma; at 2 hours 3.22 mg. After 6 hours the pre-feeding level of 0.9 mg was noted. No increase was observed following the ingestion of alanine (10 g), tyrosine (20 g), glycine (20 g), and phenylacetic acid (10 g).

Comments. The results clearly indicate that phenylpyruvic acid is present in the blood of every patient tested and that no appreciable amount may be found in non-phenylketonuric subjects. Phenylketonemia is therefore as characteristic of the disease as phenylketonuria. That the keto acid present in the blood derives from phenylalanine seems established

TABLE II. Phenylketonemic Curve Following Ingestion of Phenylalanine and Phenylpyruvic Acid in 2 Patients Treated with Benemid.

| | Hr | Blood phenylpyruvic acid: mg/100 cc plasma | |
		Before Benemid	After Benemid
DL phenylalanine (10 g)	0	.60	.96
	1	1.75	2.40
	2	2.40	3.50
	6	1.10	1.20
Phenylpyruvic acid (6 g)	0	.80	1.20
	1	5.05	6.60
	2	4.55	5.10
	6	.90	1.10

by feeding experiments. The major part of the increased amount of phenylpyruvic acid observed in the blood following DL-phenylalanine feeding apparently derives from the D form and only a minor part from the L isomer. In this respect the phenylketonuric behaves differently from the normal subject who showed no phenylpyruvic acid in the blood following ingestion of L-phenylalanine and only slight phenylketonemia following the D form. This observation would lend some support to the hypothesis of Fölling et al.(8) that the phenylketonuric organism derives phenylpyruvic acid from D-phenylalanine following conversion of the L into the D isomer. However, the hypothesis is contrary to the evidence brought forward by Prescott et al. (9) indicating that all phenylalanine present in the blood of phenylketonurics is of the L form.

Concerning the place of formation of phenylpyruvic acid from phenylalanine, it was previously suggested that most if not all keto-acid excreted in the urine might be formed in the kidneys by deamination of the excess phenylalanine present in the blood(3), but it appears probable from the present findings that this deamination process takes place also in extrarenal tissue. Since it is likely that parahydroxylation of phenylalanine is defective in the disease(10), formation of phenylpyruvic acid would represent an attempt by various tissues to dispose of the accumulating excess of phenylalanine using an alternative metabolic pathway.

Whether a difference exists between the phenylketonuric and the normal organism in the further disposal of phenylpyruvic acid is not clear from the present experiments. The phenylketonemic curve following ingestion of phenylpyruvic acid shows a similar shape in both patients and controls indicating similar mechanisms of breaking down the metabolite. However, the peak is higher in the phenylketonuric than in the normal subject approximating in the former the values seen in disorders of the liver. This would suggest that in phenylketonuria some mechanism by which the normal liver may dispose of the keto-acid is at fault. One may speculate that in normal individuals the liver is able to form phenylalanine from excess phenylpyruvic acid by amination(11), a process which might be defective in phenylketonuria owing to the accumulation of phenylalanine in the body fluids. Other known ways of disposing of the keto-acid such as urinary excretion, transformation into phenyllactic and phenylacetic acid(12), would be common to both normal and phenylketonuric subjects. If this hypothesis is correct, the disturbance of phenylpyruvic acid metabolism would be only partial in character and secondary to the error of phenylalanine metabolism. The importance of the kidney in the disposal of phenylpyruvic acid is indicated by the experiment with Benemid. Inhibition of tubular transfer of phenylpyruvic acid by the drug would easily explain the increased phenylketonemia and the decreased phenylketonuria observed in these experiments.

The correlation between phenylketonemic level and degree of mental defect is of pertinence to the still unsolved problem of the relationship of metabolic disorder to intellectual development(13). If one assumes that a hypothetic toxic action of phenylpyruvic acid is responsible for the retarded mental growth, one would expect a negative coefficient of correlation, i.e., as the I.Q. increases, phenylketonemia should decrease. Since the correlation is positive (+.434), the hypothesis is probably incorrect. The correlation coefficient for degree of nutritional state and phenylketonemia (+.47) suggests that good nutrition and, presumably, high intake of protein is often associated with high phenylketo-

nemia level. Observation of single patients seems to confirm this statistical finding; patient No. 35, for instance, who showed the highest phenylketonemic level, was a voracious eater, while patients No. 15, 33 and 34, who showed low phenylketonemia, were "feeding problems" and consumed limited amounts of food. The observed correlations for I.Q. and phenylketonemia may then depend simply on the fact that in this series, subjects with high I.Q. usually show better eating habits than low I.Q. patients.

Summary. Phenylpyruvic acid was demonstrated in the blood of 39 patients affected with phenylpyruvic oligophrenia. The values ranged from 0.31 to 1.78 mg per 100 ml of plasma. A significant increase of phenylketonemia was observed following ingestion of DL-phenylalanine, the D form causing considerably greater increase than the L isomer. Feeding of phenylpyruvic acid resulted in a sharp increase of phenylketonemia. The significance of these findings is briefly discussed.

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Nature of the Metabolic Products of C11-Cholesterol Excreted in Bile and Feces.* (19999)

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It has been generally considered that cholesterol is eliminated from the body principally as coprosterol, dihydrocholesterol and cholesterol, and that their excretion—or the excretion of their precursors—takes place across the intestinal wall(1-3). Bile has come to be regarded as a relatively minor path for the elimination of cholesterol or of its products by metabolism(4). Recent studies carried out with cholesterol-4-C14 have revealed, however, that this view is no longer tenable, for approximately 90% of the C14 of administered cholesterol-4-C14 is recoverable in bile. Furthermore, the nonsaponifiable fraction, which includes coprosterol and dihydrocholesterol as well as cholesterol, does not amount to more than 25% of the total C14 found in either bile or feces (5). Thus, by far the major part of the administered C14 is eliminated as saponifiable materials. It seemed likely that these excretion products are bile acids. Supporting evidence for this view is presented here.

Treatment of rats. Male Long-Evans rats, weighing between 250 and 300 g, were lightly anesthetized with ether. One mg of cholesterol-4-C14 containing 3 x 10^6 c.p.m. dissolved
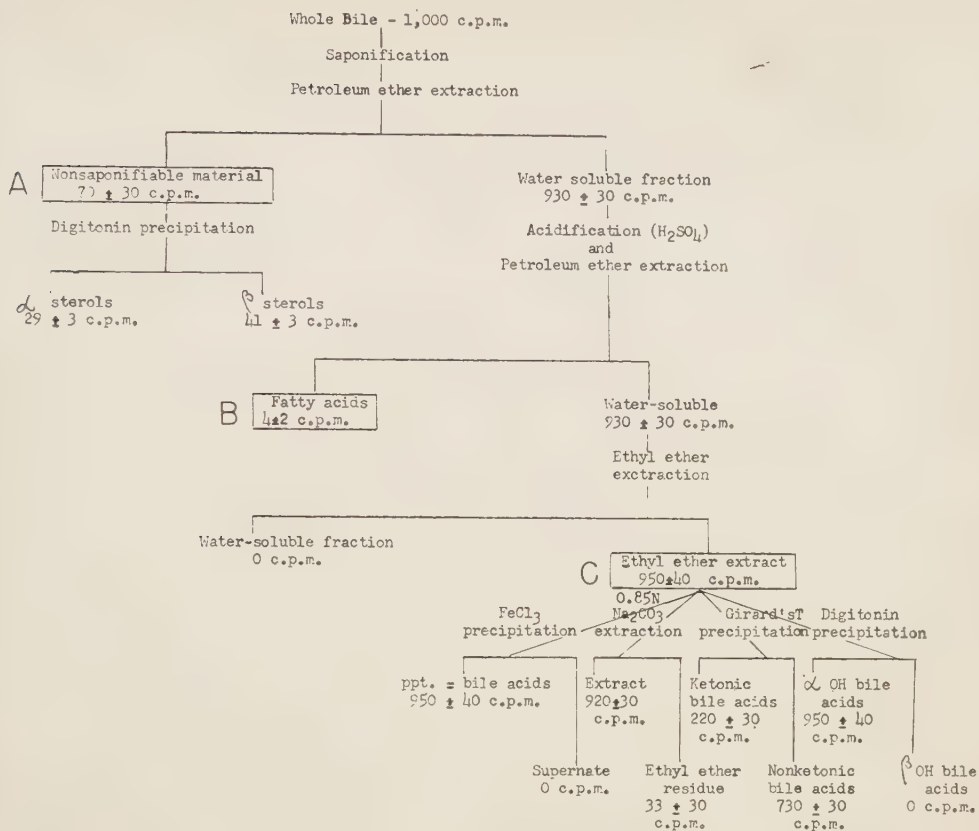
FIG. 1. Procedure for fractionation of bile. The C[14] activity in the bile samples analysed has been assigned an arbitrary value of 1000 c.p.m. The actual total activity in the samples analysed was approximately 20000 c.p.m.

in saline with the aid of Tween 20(6) was injected into their tail veins. One group of rats treated in this manner was placed in metabolism cages for the collection of feces. A second group had cannulae placed in their common bile ducts as previously described(5); these rats were kept in restraining cages. The rats were raised on a diet low in cholesterol (about 10 mg per 100 g of diet) which was also fed during the experiment.

Procedures. Fractionation. Feces were collected for 15 days after the administration of the labeled cholesterol to normal rats. In the case of the bile duct-cannulated rats, bile was collected for 48 hours. The feces samples analyzed were obtained between 72 and 120 hours. The bile samples analysed were collected from 6 to 24 hours after the administration of the C[14]. Flow sheets illustrating the fractionation procedures for bile and feces are shown in Fig. 1 and 2. *Preliminary ex-*

tractions. Feces collected for intervals of 24 or 48 hours were extracted 3 times with boiling ethyl alcohol, and the residue was transferred to a Soxhlet apparatus where it was extracted for 48 hours with ethyl alcohol. The extracts were combined and saponified as described below. It was noted that the alcohol removed all of the C[14] activity from the feces. Samples of bile were not subjected to this preliminary extraction. *Saponification.* One ml of bile or of the alcoholic extract of feces was placed in a 125 ml Erlenmeyer flask, and 6 ml of 7N NaOH were added. A bubble stopper was placed in the flask which was then autoclaved for 3 hours at 15 lb pressure. This procedure was shown in preliminary experiments to saponify taurocholic acid completely. Heating on a steam bath for a similar length of time was inadequate for this purpose. *Extraction of nonsaponifiable material.* After saponification, an equal volume of 95%
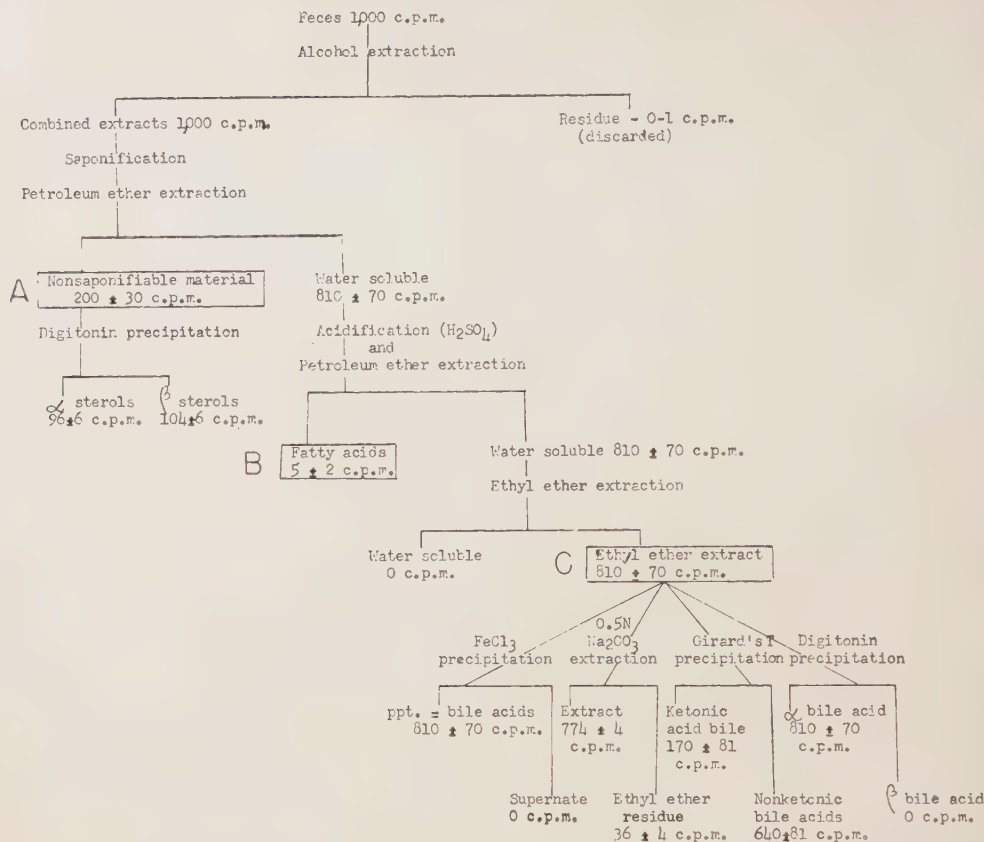
FIG. 2. Procedure for fractionation of feces. The C^{14} activity in the feces samples analysed has been assigned an arbitrary value of 1000 c.p.m. The actual total activity in the samples analysed was approximately 50000 c.p.m.

ethyl alcohol was added to the contents of the flask. An 8- to 10-fold excess of petroleum ether was next added, and the flask was vigorously shaken. The extraction with petroleum ether was repeated twice more. The extracts were then combined and concentrated on a steam bath, after which the concentrated material was transferred to a 25 ml volumetric flask. An aliquot of this nonsaponifiable Fraction A was mounted directly on an aluminum plate, and its C^{14} content determined in a flow gas counter. *Extraction of fatty acids.* The alcohol-water phase that remained after removal of the nonsaponifiable materials was then heated on a steam bath to remove the alcohol. The solution was acidified with H_2SO_4 to pH 1. Three petroleum ether extractions were carried out exactly as described for the nonsaponifiable material. These served to remove fatty acids; bile acids are insoluble in petroleum ether. *Extraction of bile acids.* The aqueous phase, which now contained neither fatty acids nor nonsaponifiable materials, was extracted 6 times with ethyl ether. This procedure removes bile acids. After 6 extractions, no C^{14} remained in the aqueous layer. The combined ethyl ether extracts were made basic with ammonium hydroxide, concentrated to a small volume, and transferred to a 25 cc volumetric flask with ethyl alcohol. An aliquot was then mounted on an aluminum plate and its C^{14} content determined. *Extractability of activity from ethyl ether extracts with 0.8 N Na_2CO_3.* An aliquot of the ethyl ether extract C was placed in a separatory funnel and extracted 3 times with equal volumes of 0.85 N Na_2CO_3. The ethyl ether was transferred to a volumetric flask and its C^{14} content determined.

Doubilet procedure for isolation of bile

acids. Another aliquot of the ethyl ether extract C containing the bile acids was taken to dryness on a steam bath. Fifty mg of cholic acid were added as carrier. The mixture was next taken up in dilute NaOH and transferred quantitatively to a 15 ml centrifuge tube. After the pH of the solution was brought to 6.8, the bile acids were precipitated as the iron salts exactly as described by Doubilet(7). The mixture was centrifuged at 2,800 r.p.m. for 15 minutes, the supernatant was decanted, and the precipitated bile acids were washed 3 times with 0.5 N Na$_2$SO$_4$. The combined supernatant and washings were evaporated to dryness, and the residue was exhaustively extracted with ethyl alcohol. The C^{14} activity of this alcoholic fraction which contained the nonbile acid activity was determined. *Ketonic bile acids.* A third aliquot of the ether extract C was used for the isolation of ketonic bile acids. They were isolated as the mercuric iodide salt of the hydrozone of the bile acids by the method of Hughes(8). The bile acids precipitated by this procedure were dissolved in pyridine, an aliquot of which was taken for C^{14} analysis.‡ *Digitonin-precipitable C^{14}.* A fourth aliquot of the ether extract C was taken to dryness, and the residue was transferred with ethyl alcohol to a 15 ml centrifuge tube. One mg of carrier cholesterol was added, and the Sperry-Webb procedure(9) for isolation of digitonin-precipitable materials followed. The washed precipitate was dissolved in methyl alcohol for determination of its C^{14} activity.

An aliquot of the nonsaponifiable Fraction A was also treated with digitonin exactly as described above.

Results. In the case of feces, we observed a difference between early and later samples in the ratios of nonsaponifiable C^{14} to total C^{14}. Some time is required to transport the C^{14}-containing bile to the excreted feces. Thus in the first 24-hour feces sample, nonsaponifi-

able C^{14} represented about 30% of the total C^{14}, whereas in feces samples collected at later intervals, the ratio fell to about 20% of total fecal-C^{14}. The higher value in the first 24-hour sample probably represents to a large extent material excreted directly through the intestinal wall, the major portion of which is nonsaponifiable. By the time the bile reaches the large intestine, the C^{14} it contributes to feces far outweighs that contributed by direct passage across the intestinal wall. Because of the above considerations, it would appear that later samples of feces present a truer picture of normal cholesterol excretion. Samples of feces collected between 72 and 120 hours after the administration of the labeled cholesterol were therefore chosen.

Bile. The distribution of the C^{14} activity is shown in Fig. 1. Little, if any, of the C^{14} activity was recovered in the fatty acid fraction.§ The nonsaponifiable fraction contained from 4 to 10% of the total C^{14} of the bile sample, slightly more than half of which was digitonin-precipitable (*i.e.*, presumably present as cholesterol, dihydrocholesterol, coprosterol). By far the major portion of the C^{14} was recovered in the ethyl ether extract of the acidified hydrolysate (Fraction C). The following evidence indicates that the C^{14} compounds in this fraction are bile acids: 1) insolubility in petroleum ether; 2) complete solubility in ethyl ether; 3) almost complete recovery in the acidic sterol fraction with the aid of 0.85 N Na$_2$CO$_3$; and 4) complete recovery of the activity in the bile acid fraction isolated by the Doubilet procedure. Since, in naturally occurring bile acids, the OH group at Carbon 3 is *trans*, none of the activity, as was to be expected, was precipitated with digitonin. About 30 per cent of the C^{14}-bile acids was found in the ketonic fraction.‡

Feces. The distribution of the C^{14} in various fractions (Fig. 2) resembled that observed for bile except that somewhat more of the activity was recovered in the nonsaponifiable fraction. The latter, of course, represents

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‡ In the course of chromatographic separation of pure bile acids it has been observed that heating a neutral solution of sodium cholate will change its R$_f$. It is possible, therefore, that the isolation procedure used here could have altered the structure of the bile acids. Thus, the ketonic bile acids may possibly be artifacts.

§ It is of interest to note that after the administration of cholesterol-26-C^{14} to rats, practically none of the fecal C^{14} was recovered in the fatty acid fraction (Fraction B, Fig. 2) (unpublished observations).

the C^{14} contribution from the intestinal wall. Thus the principal C^{14} end product in feces as well as in bile is in the nature of a bile acid.

Discussion. Evidence is presented here to show that bile acids constitute the major excretion products of cholesterol in both bile and feces. This finding is not too surprising and, indeed, was strongly indicated by the previous demonstration that, following intravenous injection of cholesterol-4-C^{14}, bile constituted the chief route of excretion of the C^{14} into the intestine, and, further, that this carbon is not recoverable as a neutral sterol(5). The loss of carbons 26 and 27, but not of carbon 4, as CO$_2$ in expired air(10) likewise is in accord with the view that a bile acid is the excretion product for the nucleus of cholesterol. Finally, the demonstration of an enterohepatic circulation of a large portion of the cholesterol metabolic end product(11) agrees well with what has long been known concerning bile acid reabsorption and reexcretion into the intestine.

The results of these previous studies on cholesterol metabolism together with the evidence presented here on the nature of the cholesterol end products now make it possible to outline the major metabolic pathway of the cholesterol molecule in the body.

Ingested cholesterol is absorbed from the intestinal tract almost entirely by way of the lymph(12), a process in which bile plays an essential role(13). Following its absorption, about 80% of the terminal 2 carbons of the isooctyl chain is gradually oxidized and eliminated as CO$_2$ in the expired air(10). A number of tissues can oxidize the chain carbon but liver is most active in this respect, and this tissue probably accounts for the greatest amount of cholesterol destroyed in the body (6). Following the removal of the terminal carbons of cholesterol, and other less drastic alterations, the residue is excreted into the bile as bile acids. The remaining 15 to 25% of the cholesterol is eliminated either through bile or across the intestinal wall as neutral sterols containing 27 carbons.

A large portion of the bile acids excreted into the intestine is promptly reabsorbed into the body via the portal blood. These bile acids are then quickly reexcreted through the bile only to be reabsorbed a second time and again excreted. This enterohepatic circulation of carbon 4 of bile acids undoubtedly continues through many cycles but, with each cycle, a small amount of bile acid escapes to be eliminated in the feces. The total of these small increments of escaping bile acids finally represents between 75 and 85% of the fecal sterols. This, in addition to the 27-carbon containing sterols eliminated either directly through the intestinal wall or through the bile accounts for almost all of the cholesterol excreted from the body.

Summary. Cholesterol-4-C^{14} was administered intravenously to normal rats and the C^{14} end products in feces and bile were fractionated. 1. From 4 to 10% of the total C^{14} in bile and from 17 to 23% of fecal C^{14} were recovered in the nonsaponifiable fraction which includes cholesterol, dihydrocholesterol, coprosterol, etc. 2. Little if any of the C^{14} in either bile or feces was recovered in the fatty acid fraction. 3. Evidence is presented to show that about 80% of the fecal C^{14} and about 90% of the bile C^{14} are present in the form of bile acids. 4. The major metabolic pathways for the nucleus carbons of cholesterol are outlined.

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Cross Resistance to Antibiotics: Effect of Exposures of Bacteria to Carbomycin or Erythromycin *in Vitro*.* (20000)

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Among the antibiotics currently in wide use, cross resistance to one antibiotic resulting from repeated or prolonged exposures to another has been demonstrated to occur between aureomycin and terramycin(1-5), between either of these agents and chloramphenicol (1-4), and between streptomycin and neomycin(3,6). In the course of studies originally designed to investigate some bacteriological aspects of the new antibiotic carbomycin (Magnamycin)(7) and its clinical potentialities, it became apparent at once that the antibacterial spectrum of this new agent closely parallels that of erythromycin, both qualitatively and quantitatively. A study of cross resistance between these 2 agents was, therefore, undertaken. In this paper are reported the results of parallel tests for sensitivity of a number of bacterial strains to carbomycin and erythromycin together with the results of tests for resistance and cross resistance to these agents resulting from repeated subcultures of bacteria in increasing concentrations of each of these antibiotics.

Materials and methods. Both the erythromycin and the carbomycin used in this study were obtained in the form of the purified crystalline base; the labeled potency was 890 μg/mg of erythromycin and 830 μg/mg of carbomycin. The methods used were similar to those employed in similar studies previously reported from this laboratory(3,6,8). The tests for sensitivity were done by an agar-plate dilution method (10% horse blood was incorporated in the agar throughout this study) and the attempts to increase resistance were made by serial subcultures of each strain

from the agar plate containing the maximum concentration of antibiotic on which good growth occurred in the sensitivity test to another series of agar plates containing similar 2-fold serial dilutions of the antibiotic. For some of the cross resistance tests with carbomycin, a number of the strains used had been developed in other studies(8) by subcultures on graded concentrations of penicillin, streptomycin or erythromycin and also with combinations of penicillin and erythromycin(8) and of streptomycin and erythromycin (8). In a number of the latter studies, some of the serial subcultures had been made daily, and broth was used instead of agar for the subcultures of some of the strains(8); in the present study the subcultures on carbomycin agar were made after 48 hours of incubation in each instance. In this study the sensitivity of a strain to erythromycin is expressed as the minimum concentration of antibiotic in μg of the active base per ml of media which completely inhibits visible growth in 24 hours (M.I.C.); in the case of carbomycin, M.I.C. is expressed in terms of actual weight per ml.

Results. Sensitivity of bacteria to carbomycin and erythromycin. Parallel tests for sensitivity to carbomycin and erythromycin were carried out on 74 strains of a large variety of bacterial species. Some of these organisms were stock laboratory strains, but the great majority were freshly isolated from patients with acute infections. The results are listed in Table I. It is seen that the gram-positive organisms and the gram-negative cocci were all highly sensitive and the strains of *Hemophilus* were moderately sensitive while the coliform and the enteric bacilli were all resistant to both carbomycin and erythromycin. Of particular interest, however, is the fact that, with the exception of 3 strains which were equally sensitive to both agents, all of the sensitive strains required greater concentrations of carbomycin than erythromycin to

produce the same degree of inhibition; of the 55 sensitive strains, 45 required 4 or 8 times as much carbomycin as erythromycin; 5 required 16 times as much and 2 required twice

TABLE I. Sensitivity of 74 Bacterial Strains to Carbomycin and Erythromycin.*

Organism			M.I.C.,† µg/ml	
			Carbo-mycin	Erythro-mycin
Pneumococcus, type	5		.2	.05
	7		.4	.05
	10		.4	.05
	17		.4	.05
	18		.4	.05
	18		.4	.05
	19		.1	.02
	4		.4	.05
	18		.1	.02
	5		.1	.02
	10A		.2	.02
	19		.8	.1
*Streptococcus C203, Group		A	.2	.05
*	98	A	.1	.02
		A	.8	.05
		B	.8	.2
*	C3	D	.8	.2
*	D76	D	.8	.8
		D	6.3	6.3
		D	.4	.05
		D	1.6	.4
		D	1.6	.4
*	K131	E	.8	.05
*	166B	G	.4	.05
*	167B	G	.4	.05
Microaerophilic streptococcus		{	.8	.1
		{	.4	.1
Streptococcus mitis		{	.2	.05
			.4	.05
			.4	.05
			.4	.1
			.4	.05
			.4	.05
			.8	.05
			.4	.05
			.4	.1
			.4	.1
Staphylococcus albus			6.2	1.6
aureus		{	1.6	.4
			3.1	.4
			1.6	.4
			1.6	.4
*Sarcina lutea			.1	.02
*Bacillus cereus No. 5			1.6	.4
Diphtheroid		{	.8	.05
		{	,,	,,
Neisseria meningitidis, 2A		{	.2	.1
		{	.4	.4
			.8	.4
gonorrhoea		{	1.6	.4
		{	,,	,,
Haemophilus influenzae		{	12.5	3.1
			50	12.5
			50	12.5
		{	6.3	.8

Organism			M.I.C.,† µg/ml	
			Carbo-mycin	Erythro-mycin
Escherichia coli			200	100
,,	,,			100
,,	,,		>200	>200
Aerobacter aerogenes				200
,,	,,			
,,	,,			>200
Klebsiella pneumoniae,		A		
,,	,,	A	200	200
,,	,,	A		
,,	,,	B		
Pseudomonas aeruginosa				
,,	,,			
,,	,,		>200	>200
,,	,,			
,,	,,			
Salmonella salinatis				
,,	manhattan			
,,	st. paul			
,,	oranienberg			

* Indicates stock strains; the numbered strains of groups D, E and G streptococci were obtained from Dr. Rebecca Lancefield; all other strains were freshly isolated from patients in the hospital by Miss Marion E. Lamb.

† Minimum complete inhibiting concentration.

as much carbomycin as erythromycin for complete inhibition.

Cross resistance between carbomycin and erythromycin. Five of the strains which had been made resistant to erythromycin in the previous study of Haight and Finland(9), together with the corresponding parent strains which had been transferred in parallel on antibiotic-free agar were tested simultaneously for their resistance to carbomycin and erythromycin. The same 5 parent strains were also subjected to a second series of parallel subcultures on antibiotic-free agar and on agar containing increasing concentrations of carbomycin; after 20 such subcultures, all of the strains of the second series were likewise tested at the same time for their sensitivity to carbomycin and erythromycin. The results are shown in Table II, and the progressive increases in resistance to carbomycin resulting from the repeated transfers in the presence of the homologous antibiotic are shown in Fig. 1. Marked increases in resistance to carbomycin developed in each of the strains which had been repeatedly subcultured in the presence of that antibiotic. The strains which had been repeatedly exposed to erythromycin, with the exception of Strep. C203, increased simi-

TABLE II. Cross Resistance between Carbomycin and Erythromycin.

Strain	M.I.C.,* µg/ml	
	Carbo-mycin	Erythro-mycin
Series 1†		
Staphylococcus 192 — 0 ‡	3.1	.4
ER	>800	>800
193 — 0	1.6	.4
ER	>800	>800
Enterococcus — 0	3.1	.4
ER	200	>800
Streptococcus mitis — 0	.1	.05
ER	1.6	.4
C203 — 0	.2	.05
ER	.4	.1
Present series (Fig. 1)		
Staphylococcus 192 — 0	3.1	1.6
CR	400	100
193 — 0	3.1	1.6
CR	>800	100
Enterococcus — 0	3.1	.2
CR	50	3.1
Streptococcus mitis — 0	.2	.05
CR	25	.2
C203 — 0	.2	.05
CR	6.3	.8

* Minimum complete inhibiting concentration.
† See Haight & Finland(9), Fig. 1.
‡ 0 = repeated subcultures on antibiotic-free media; ER = after repeated transfers on erythromycin; CR = after repeated subcultures on carbomycin.

FIG. 1.

larly in resistance to the homologous agent. Cross resistance between the carbomycin and the erythromycin was complete in every instance. Moreover, the ratio of the minimum complete inhibiting concentration of carbomycin to that of erythromycin for each of the parent strains and for their resistant variants was essentially similar to that observed in Table I, except in the case of the carbomycin-resistant strain of *Streptococcus mitis* which required 128 times as much carbomycin as erythromycin. *Additional tests* for sensitivity to carbomycin and erythromycin were carried out simultaneously with 7 strains of staphylococci and the variants of these strains derived by serial subcultures in the presence of erythromycin, streptomycin or both in combination(8); similar tests were also done with 5 additional strains of staphylococci and their respective variants derived by serial transfers on erythromycin, streptomycin, or a combination of both. The results are given in Table III. Each of the parent strains and their variants required 4 to 16 times as much
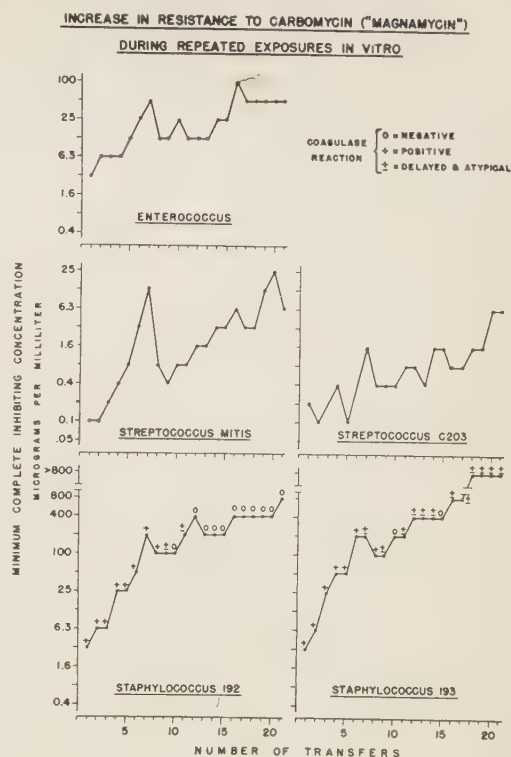
carbomycin as erythromycin to produce complete inhibition. Increases in resistance to erythromycin was accompanied, in every instance, by a parallel increase in resistance to carbomycin.

Effect of development of carbomycin resistance on coagulase production by staphylococci. The results of coagulase tests carried out daily during the successive transfers of 2 staphylococcal strains in increasing concentrations of carbomycin are shown in Fig. 1. *Staph. 192* lost its coagulase activity between the 9th and 12th transfer in the presence of carbomycin; in the case of *Staph. 193,* on the other hand, the coagulase activity became altered at about the same stage of the transfers and, except on 2 occasions, this activity was reduced and delayed in appearance but still demonstrable. In previous studies, coagulase tests done during or after successive exposures of other strains of staphylococci to erythromycin yielded irregular results. In the studies of Haight and Finland(9) on 4 strains, including the 2 just mentioned which are shown

TABLE III. Cross Resistance between Erythromycin and Carbomycin.*

A. Effect of repeated exposures to penicillin and/or erythromycin

Strain	Min complete inhibiting conc., µg/ml							
	Erythromycin				Carbomycin			
	0	P—R	E—R	P+E—R	0	P—R	E—R	P+E—R
Staphylococcus 1			.4	.2		3.1	3.1	3.1
2			25	.8		3.1	100	25
3			6.3	3.1		3.1	100	50
4	.4	.4	800	3.1	6.3	6.3	100	25
5			200	1.6		6.3	>400	12.5
6			100	>800		6.3	>400	>400
7			6.3	.8		12.5	100	6.3

B. Effect of repeated exposures to streptomycin and/or erythromycin

Strain	0	S—R	E—R	S+E—R	0	S—R	E—R	S+E—R
Staphylococcus S			12.5	6.3		3.1	50	25
W			12.5	.8		3.1	50	3.1
M	.4	.4	6.3	6.3	3.1	3.1	25	25
V			>100	6.3		6.3	>100	25
A			12.5	1.6		3.1	50	6.3

* Strains used in study of Purcell, Wright and Finland(8).
 0 = no previous exposure to antibiotics; R = previous transfers with antibiotics; P = penicillin; S = streptomycin; E = erythromycin.

in Fig. 1, the coagulase producing activity in the case of each strain first became poor and then was completely lost during the successive subcultures with erythromycin. In subsequent studies(8), other strains of staphylococci which increased in resistance during repeated transfers in the presence of erythromycin generally retained their coagulase activity apparently unaltered. Other distinctive properties of the staphylococci were not demonstrably altered during the course of their repeated exposures to carbomycin. The basis of these differences in effects on coagulase activity is not clear.

Discussion. The data presented in this paper indicate a very close relationship between carbomycin and erythromycin with respect to their antibacterial activity; this relationship is both qualitative and quantitative. It appears from these data that, weight for weight, erythromycin is from 4 to 16 times as active as carbomycin against almost all of the bacterial strains for which the sensitivity to these agents has been compared. High grades of resistance to carbomycin and erythromycin develop with considerable ease and rapidity during the course of repeated subcultures of susceptible staphylococci and of certain streptococci in the presence of each of these antibiotics. Moreover, increases in resistance resulting from exposures to the homologous antibiotic are accompanied by increases in resistance of about the same order of magnitude to the other agent to which the organisms had not previously been exposed. These findings and the claims that each of these antibiotics are active against rickettsias and against the psittacosis and related viruses (7,10) suggest the possibility that the 2 agents may be chemically very closely related in spite of the apparently divergent physical constants presented in the original papers describing the discovery of erythromycin(10) and carbomycin(7); the data, however, do not constitute valid proof of such a chemical relationship. The comparisons of the biological activities of these 2 substances are reminiscent of the comparative activities of aureomycin and terramycin, these antibiotics have many distinctive physical characteristics but they have recently been shown to have very similar basic chemical structures(11,12).

Summary and conclusions. The antibacterial spectrum of carbomycin parallels very closely that of erythromycin. Both of these new antibiotics are highly active against gram-positive and gram-negative cocci, moderately active against strains of *Haemophilus* and are essentially inactive against coliform and enteric bacilli. Against susceptible bacterial strains erythromycin is usually from 4 to 16 times more active than carbomycin, weight for

weight. Repeated subcultures of staphylococci and of certain strains of streptococci in the presence of increasing concentrations of either one of these antibiotics result in fairly rapid and marked increases in resistance not only to the antibiotic to which it was exposed but to the other agent as well.

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Role of the Pancreas in Prevention of Fatty Liver in the Hypophysectomized-Thyroidectomized Dog.* (20001)

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The pancreas, by virtue of its exocrine function, exerts a profound influence on the liver. This has been clearly brought out in the case of the fatty liver that appears in the completely depancreatized dog receiving insulin(1). It is shown in this report that the digestive function of the pancreas may be implicated in the development of another type of fatty liver, namely, that observed in the hypophysectomized - thyroidectomized (HT) dog. This type of fatty liver is prevented if choline in the free form is added to the diet (2). Evidence presented here shows that extra methionine is also efficacious. That the fatty liver in the doubly-operated dog is the result of pancreatic insufficiency is indicated by the additional finding that its development can be prevented by feeding raw pancreas.

Experimental.· Nine dogs were used in this study. Throughout their stay in the laboratory they were fed, daily, 30 g of lean meat per kg of body weight, in addition to sucrose, vitamins, and a salt mixture. The exact com-

position of this diet has been described elsewhere(3). The pituitary glands were first removed, and one to 2 weeks later the thyroids were excised. The animals were then divided into 3 groups (Table I). The control dogs (Table I) were fed just the lean meat diet described above. The second group was fed, in addition, 25 g of pancreas with each meal. The third group was fed 2 g of methionine with each meal. To ensure the exact dietary intakes recorded for each dog, forced feeding was resorted to at times. All of the dogs were maintained for not less than 749 days. This period exceeds the minimum time necessary for the consistent development of the fatty liver in HT dogs(2). At the end of the period of observation, livers and blood were removed, and their lipide contents determined by methods previously described(4).

Results. In a preceding paper it was shown that hypophysectomy and thyroidectomy result in the development of a fatty liver in the dog in 217 to 419 days(2). In the present study the livers of the 2 control dogs contained 21.6% and 27.4% total fatty acids (Table I). From 2 to 3% total fatty acids were found in the livers of the dogs fed pan-

TABLE I. Plasma and Liver Lipides of HT Dogs Fed Pancreas and Methionine.

Dog No.	Body wt, kg At hypophysectomy	Body wt, kg At thyroidectomy	Body wt, kg Final	Period maintained after hypophysectomy-thyroidectomy, days	Supplement fed Type	Supplement fed Per meal,* g	Plasma total lipide, mg %	Liver lipides Wt, g	Liver lipides Total fatty acids, % wet wt
HT-47	6.5	6.7	8	749	0, control	—	946	290	21.6
49	7	7.6	8.5	749	0, "	—	782	268	27.4
34	7.5	9.4	9.2	826	Pancreas	25	798	260	3
37	6.3	8.7	9.2	826	"	25	1349	278	2.3
48	7	7.2	11.7	750	"	25	945	300	2
50	6.5	6.6	15	750	"	25	2255	405	2
40	8	8.4	10.7	838	Methionine	2	1395	406	2.4
41	6	7.9	8.8	838	"	2	1346	266	1.7
42	8.7	9.6	16.7	838	"	"	1675	652	3.8

* The dogs received 2 meals per day.

creas in addition to the regular meat diet. The livers of dogs fed methionine contained from 2 to 4% total fatty acids.

The values for total plasma lipides are also recorded in Table I. They ranged from 780 to 2260 mg %. The concentration of total lipides in plasma of normal dogs fed the lean meat-sucrose diet does not exceed 750 mg % (5). As in previous studies, all of the HT dogs showed a considerable increase in weight (Table I). This increase was not unexpected because forced feeding was employed to maintain the preoperative caloric intake.

Discussion. The amounts of lean meat fed each HT dog contained approximately 0.5 g of choline and 3 g of methionine, and it is important to recognize that such amounts of lipotropic substances more than suffice to prevent the development of fatty livers in *normal* dogs(1). Our earlier finding, that the addition of a lipotropic substance to the lean meat diet prevents fatty livers in HT dogs, is fully confirmed in the present study with methionine. It was this type of action that led us to suggest that the fatty liver in the HT dog resulted from 1) an interference in the mechanism for release of lipotropic factors from the ingested meat in the intestine and/or 2) defective absorption of these substances. It is well established that the rate of carbohydrate absorption from the intestine is depressed after thyroidectomy(6) and hypophysectomy(7), and it is therefore conceivable that the absorption of free lipotropic substance from the intestinal tract is also depressed. The observation, however, that the feeding of 25 g of raw pancreas per meal is also effective suggests rather that the excision of both thyroid and pituitary glands interferes with the release of lipotropic factors from ingested meat in the intestinal tract. The presence of an intact pancreas in the HT dog is not incompatible with this view since it is reasonable to infer that the exocrine portion of the pancreas, a site of rapid protein synthesis, is functionally depressed by hypophysectomy and thyroidectomy.

Summary. 1. Dogs deprived of both thyroid and pituitary glands develop fatty livers. The livers of 2 such dogs that were fed a diet rich in lean meat for 750 to 840 days contained 22 and 25% fatty acids. 2. The addition of 2 g of methionine to each meal completely prevented the development of fatty livers in HT animals so fed. 3. The addition of 25 g of pancreas to the daily food intake also prevented the fatty livers. 4. The factors responsible for the accumulation of fat in the livers of hypophysectomized-thyroidectomized dogs are discussed.

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AUTHORS' INDEX
VOLUME 81

(The numerals indicate the page)

SUBJECT INDEX
VOLUME 81
(The numerals indicate the page)